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Erle S. Robertson *Editor*

Microbiome and Cancer

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Preface

Studies targeted to understanding our interactions with the microbial world have been ongoing for more than a century. This was demonstrated through the initial link between infectious agents and cancer, as identified by Peyton Rous. He showed an association between a filterable agent and development of sarcomas in chickens, in 1911. This agent was identified as the Rous sarcoma virus and was shown to be transferrable to birds that were disease free. Approximately five decades later, the first human oncogenic virus was identified by Anthony Epstein and Denis Burkitt, with colleagues Yvonne Barr and Bert Achong, in 1964 at the Middlesex Hospital in England. This certainly changed our understanding of the contributions of infectious agents to the cancer phenotype many years after the discovery of the first link between cancer and the RSV agent in avian species. Today there have been increasing associations with infectious agents and human cancers from viruses to parasitic agents. In fact, two of the most impressive successes in the cancer vaccine arena have been against viral agents, as seen with vaccines against the hepatitis B virus (HBV) and the human papilloma virus (HPV). The effectiveness of these vaccines in reducing the incidences of hepatocellular cancer (HCC) and cervical cancer, respectively, demonstrates the importance of understanding the links between pathogenic infectious agents and cancer.

Today approximately 20% of all known cancers are associated with infectious agents as major drivers of the pathology. This is likely to be an underestimate as the technological hurdles become more manageable and sensitive in detecting these agents in the cancer tissue; it is likely that this would increase. The discoveries of these associations were supported by strong epidemiological evidence, which has been substantiated by multiple studies. More recently, there were more studies which showed that the contributions of microorganisms do not necessarily have pathogenic consequences but can also be beneficial and in some may provide protective contributions.

The era of the microbiome has given us additional ammunition as to the importance of microorganisms in our daily activities and has shown that homeostasis of our microbial flora is critical to our overall well-being. The large number of investigations into the microbiome at different anatomical sites has demonstrated that the

specific sites of the human body have a preferential microbiome and that changes can lead to the establishment of dysbiosis at these sites resulting in inflammation. This in addition to the direct activities of these agents can function as triggers for proliferation.

This is a complex line of investigation and we now know a great deal more compared to a decade ago. These studies have also provided clear insights into the complex molecular systems, which link microbial homeostasis with inflammation and metabolism, and are based on the physiologic activities between host cells and the microbes that they are associated with in the particular microenvironment. It is also becoming more acceptable due to the plethora of studies to understand the changes in the gut microbiome that different treatment modalities can induce a range of comorbidities in addition to the cancer being targeted. The fact that treatment of cancer patients with chemotherapeutic agents, radiation, and broad-spectrum antibiotics can change the normal microbiota, therefore predisposing the patients to colonization with pathobionts, provided important information as to ways to curb the related comorbidities. Importantly, these changes are likely not only in the gastrointestinal tract but may also affect the microbiota at different anatomical sites. Understanding the changes, which occur, will certainly shed light on potential avenues for interventions.

This initial book is an attempt to address the limited focus on the microbiome associated with the broad range of different cancers along with their microenvironment, and is certainly not comprehensive. I would like to thank the contributors for their time and efforts in attempting to address the more focused area of study related to the microbiome and specific cancers. One major issue we had in assembling this book was that many potential authors were dealing with time constraints and funding so that they were not able to find the time to contribute. Therefore, I am indebted to the ones who found the time from their busy schedule to write the chapters, which are included here. In a time when we are all constrained for time and balancing many other commitments, setting aside the time was a true labor of love. Certainly, there are areas which we have missed due to these constraints, and we hope that in another period, in the future, we would be able to deliver a more comprehensive text as the field becomes more mature. Nonetheless, I believe that the current volume approaches this complex subject area with a wonderful series of chapters. Readers who are novices in the field of microbiome and cancer, as well as more experienced investigators, would find them enlightening. It would certainly be helpful for the many trainees in graduate school or medical school who would like to obtain information that is more concise and focused in this particular area.

As additional studies continue to investigate the cancer-associated microbiome, the differences that will likely exist in the gut microbiota compared to the tumor microbiota will be illuminated. One would expect that there would be some overlap between the gut microbiota and the tumor microbiome in terms of the identified microbiota. However, as more studies related to the tumor microbiome (oncobiome) provide additional data, it will show that, as expected, the volume of microorganisms in the GI tract is much higher than that seen in the tumor microenvironment. Nevertheless, these microorganisms may contribute to the initiation, development,

and maintenance of the tumor microenvironment. They may also be opportunistic, in that the tumor microenvironment would be a perfect place for survival, and may vary based on the oxygen gradient of the tumor, with different levels of hypoxia. The contributions of the entire microbial milieu may also be complimentary. The combined signaling may synergistically drive proliferation and influence survival of the tumor. Clearly, some organisms may have protective influences compared to others, which may be deleterious to the host. This provides a glimpse into the stringent balance that exists in the microenvironment, important for long-term homeostasis.

We have 17 chapters that include the skin microbiome and viruses, microbes associated with glioblastomas, the breast cancer microbiome, ovarian cancer and associated microbiota, the microbiome and lung cancer, infection-induced hepatocellular carcinomas, and manipulation of the host immune system by small DNA tumor viruses. Additionally, we have chapters covering the immune recognition in intestinal cancers, metabolites in promoting and preventing cancer, the virome in hematologic malignancies, esophageal carcinomas and infectious agents, head and neck cancers and infections contributing to its development, mesotheliomas and SV40 infection, and vaccine strategies. Some of these areas are still developing fields, and so we would expect that more information would become available in the near future that would provide greater insights into the role of the oncobiome in cancer.

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Chapter 1
Microbiome and Human Malignancies

Abhik Saha and Erle S. Robertson

Abstract Recent technological advances have revolutionized our current understanding of the role of human microbiota in cancer development. Several high-throughput Next Generation sequencing studies including metagenomics and transcriptomics data, along with microarray-based technologies suggest that dysbiosis in the commensal microbiota can initiate a number of inflammatory syndromes as well as multiple cancers in humans. Immune deregulation by the microbial community is considered one of the major contributing factors for cancer development. In this chapter, we broadly discuss recent developments in understanding the interaction of human microbiome and its contribution to cancer, and the possibilities of future diagnostic, as well as potential for development of targeted therapeutics.

Keywords Microbiota · Cancer · Next-gen sequencing · Metagenomics · Transcriptomics · Microarray

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1.1 Introduction

Cancer is one of the leading causes of morbidity and mortality worldwide, contributing nearly one in every six deaths. As the human lifespan increases, the complexity as well as the incidence of the disease also increases. According to the World Health Organization (WHO) the number of new cases is expected to be amplified by approximately 70% over the next two decades [1]. Out of many established cancer associated factors, microbial infections over the last 100 years have been shown to contribute to nearly 20% of all human cancers, equivalent to close to two million new cases per year [2, 3]. Among the microbial community, viruses are so far the best-studied component for their role in cancer development. These viruses include Epstein-Barr virus (EBV or HHV4), hepatitis B virus (HBV), human papilloma virus (HPV), hepatitis C virus (HCV), human T-cell leukaemia virus (HTLV-1), Kaposi Sarcoma associated herpesvirus (KSHV or HHV8) and the recently discovered Merkel cell polyomavirus (MCPyV) [4, 5]. It is well established that in case of some cancers viral infection appears to be absolutely necessary, such as, HPV infection in the development of anogenital cancers or hepatitis virus (HBV and HCV) infections in hepatocellular carcinoma (HCC) [6, 7]. These have a direct role in driving these cancers as primary contributors. However, it is not yet fully established why some individuals infected with tumor viruses do not develop cancer over their entire lifetime. For example, the majority of the world population (>95%) has been shown to be asymptomatically infected with EBV, the first known human tumor causing virus [[8] and reviewed in [9]]. However, these human tumor viruses drive the development of cancers when the immune system is compromised, exemplified as organ transplant or HIV infected individuals (AIDS patients), and are therefore opportunistic in nature [10].

Although viruses had long been identified as major cancer causing agents, our understanding of the extent of this problem connecting other microbes including bacteria, archaea, fungi and even parasites began only in recent decades and has continued to expand. A growing body of evidence indicates that microbes can play a much larger role in the development of several human malignancies, and indicates the limited understanding of their overall role we have today (reviewed in [11, 12]). For example, recently studies have shown that perturbation of the microbial community (referred to as “dysbiosis”) significantly impairs the response to cancer therapy [12]. Thus, an optimal response to cancer therapy requires an intact commensal microbiota, which regulates the tumor microenvironment through inflammatory cytokines and reactive oxygen species (ROS) production [13].

The microbial kingdom, including bacteria, viruses, archaea, fungi and protists have coevolved with the human system for many years, resulting in intricate host-microbiome interactions and in turn influences a number of physiological pathways—particularly affecting the host immune system [14]. As a result, disruption of the microbiota contributes to a variety of human diseases including immune disorders and cancers (Fig. 1.1) [2, 11, 12, 14]. Cumulative data generated over many decades has enhanced our understanding of the major role that viruses play in devel-

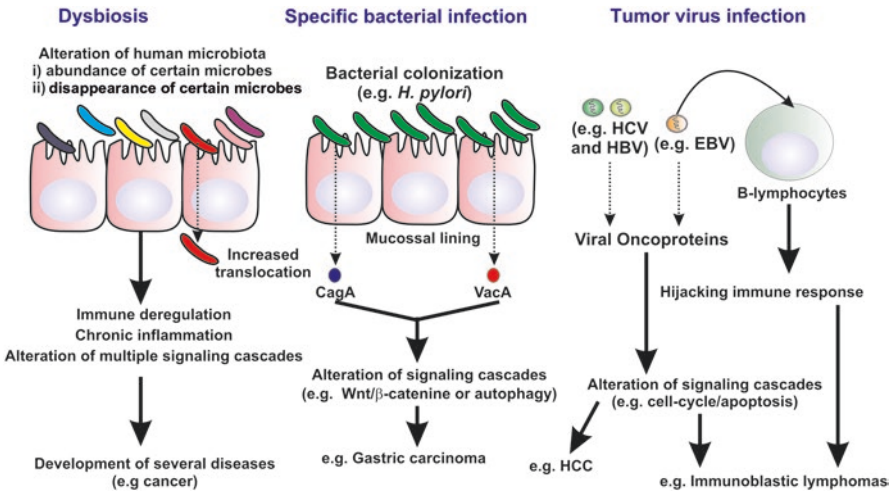

Fig. 1.1 Mechanisms by which microbes promote cancer. Several environmental factors such as diet, cigarette smoking, alcohol consumption, drug treatment and insanitary habits along with genetic predispositions promote 'dysbiosis'—an alteration of physiologic microbiota leading to a number of pathological conditions, including cancer. The alteration of microbiota severely deregulates the host immune response thereby promoting cancer development. Moreover, infections and subsequent colonization of a specific bacterium (e.g. *H. pylori* infection in stomach mucosal epithelial lining) or a virus (e.g. HBV and HCV infections in hepatocytes or EBV infection in B-lymphocytes) through employing their virulence factors, toxins or oncoproteins can also significantly modulate multiple cellular signaling pathways (e.g. *H. pylori* encoded protein CagA activates Wnt/β-catenin signaling whereas VacA blocks autophagy), which in turn lead to development of several human cancers

opment of a number of cancers [5, 15]. While the functions of achaea and fungi in the neoplastic process are largely undefined, a number of recent studies indicated an obvious bacterial association with several human cancers [12]. Of note, *Helicobacter pylori* (*H. pylori*), an early example of an individual member of bacterial community associated with the development of gastric cancer, failed to develop cancer in a germfree mice model [16]. This suggests that *H. pylori* infection alone is not sufficient for cancer development; and that participation of other microbial members appeared to play an important role in the onset of this cancer. On the other hand, in some cases an entire microbial community was shown to promote cancer propagation, such as the transmissible nature of a microbial community in the development of colorectal cancer. In addition, treatment with broad-spectrum antibiotics demonstrated promising outcomes in cancer therapy. Despite recent rapid advances in identifying entire tissue microbiota, delineating the major cancer-causing organism within the microbial community still remains a key challenge in this field. Currently, the field is largely focused on defining the underlying molecular mechanisms governing microbial interactions. A key direction for the field is to identify functional relationships between different microbial kingdoms and the interplay between the tissue specific microbiota (such as, gut microbiome), and multiple cellular pro-

cesses and pathways (such as, the immune system) [17–21]. In this chapter, we will discuss recent development into our current understanding of the overall contribution of different microbial agents in cancer propagation and the opportunity to enhance both diagnosis and therapy.

1.2 Technological Advancement In Lieu of Microbes Associated Cancers

Until recent years, the advent of high-throughput DNA sequencing and microarray technologies have radically changed our perspective regarding the overall infectious/causative agents associated with human cancer development (Table 1.1) (reviewed in [30–32]). These technologies, such as 'Metagenomics', led us to identify the entire microbial pool and the relative abundance of individual members within that milieu (Fig. 1.2). Metagenomics is a powerful tool to understand the human microbiota, describing the diversity of the microbial kingdoms and trans-kingdom interactions [33]. However, metagenomics of a complex biological sample is incapable of revealing gene expression patterns both of host and parasite origin in order to pinpoint functional dysbiosis in the course of development of several human diseases, including cancer. In addition, a significant proportion of the metagenomics data remain un-utilized due to lack of proper reference genomes in the database [34]. For example, more than 80% of the viral DNAs lack reference sequences [35]. Moreover, it is difficult to categorize and maintain the accuracy of the vast amounts of information derived from the moderately short genomic fragments generated by next-generation sequencing, which can result in erroneous annotation. Additionally, the high level of contamination of the human genome is another challenge faced during metagenomics experiments [36]. Nevertheless, through employing metagenomics technology scientists were able to discover Merkel cell polyomavirus (MCPyV), the latest addition in the list of human tumor viruses, in 2008 [4]. A combinatorial approach of various meta-omics including metagenomics, meta-transcriptomics, meta-proteomics and metabolomics can certainly help us understand the precise role of the human microbiome and thereby provide novel strategies for disease management [37]. For example, our group has developed a microarray-based approach (termed as 'PathoChip') containing 60,000 probes for simultaneous detection of both forms of nucleic acids (DNA and RNA) representing all known viruses, 250 helminths, 130 protozoa, 360 fungi and 320 bacteria which are known pathogens. The 'PathoChip' consists of two distinct set of probes—firstly, the 'unique' set of probes for each identified virus, and secondly the 'conserved' set of probes targeting genomic regions that are well conserved between members of a family of viruses, thereby allowing us to detect previously uncharacterized microbial agents. Since the PathoChip technology involves an amplification step, it allows detection of various microorganisms that are present in low genomic copy numbers in tumor samples, or which were fragmented during sample

Table 1.1 Microbiota associated with different cancers[a]

Cancer types	Associated microorganisms	Experiment	Reference
Colorectal cancer	Enriched: Fusobacterium species, Selenomonas, and Leptotrichia species, Enterobacteriaceae, Methanobrevibacter (Archaea, Methanobacteriales), Bacteroides, Roseburia, Ruminococcus, Oscillibacter, Peptostreptococcus, Parvimonas	Metagenomics	[22–24]
Prostate cancer	Enriched: Propionibacterium acnes		[25]
Breast cancer	Enriched: Bacillus, Enterobacteriaceae, Staphylococcus, Comamonadaceae Reduced: Prevotella, Lactococcus, Corynebacterium, Streptococcus, Micrococcus		[26]
Skin cancer	Enriched: Merkel Cell Polyomavirus (MCPyV)		[4]
Acute myelogenic leukemia (AML)	Enriched: Rhizomucor pusillus (zygomycetous fungus)	Microarray	[27]
Triple negative breast cancer (TNBC)	Enriched: Viruses: Herpesviridae, Retroviridae, Parapoxviridae, Polyomaviridae, Papillomaviridae, Bacteria: Arcanobacterium, Brevundimonas, Sphingobacteria, Providencia, Prevotella, Brucella, Escherichia, Actinomyces, Mobiluncus, Propionibacteria, Geobacillus, Rothia, Peptinophilus, and Capnocytophaga Fungus: Pleistophora, Piedra, Fonsecaea, Phialophora and Paecilomyces Parasite: Trichuris, Toxocara, Leishmania, Babesia and Thelazia		[28]
Ovarian cancer	Enriched: Viruses: Yaba Monkey tumor virus, Yaba-like disease virus, Monkeypox virus, Myxoma Virus, human papilloma viruses, herpesviruses Bacteria: Brucella, Chlamydia and Mycoplasma Fungus: Aspergillus, Candida, Rhizomucor, Cladosporium, Acremonium, Alternaria, Cryptococcus, Pneumocystis, Coccidioides, Trichosporon, Malassezia, Rhodotorula, Geotrichum Parasite: Dipylidium, Trichuris, Echinococcus, Strongyloides, Trichinella, Schistosoma, Leishmania, Ascaris, Trichomonas		[29]

[a]The data were derived from various metagenomics and microarray experiments

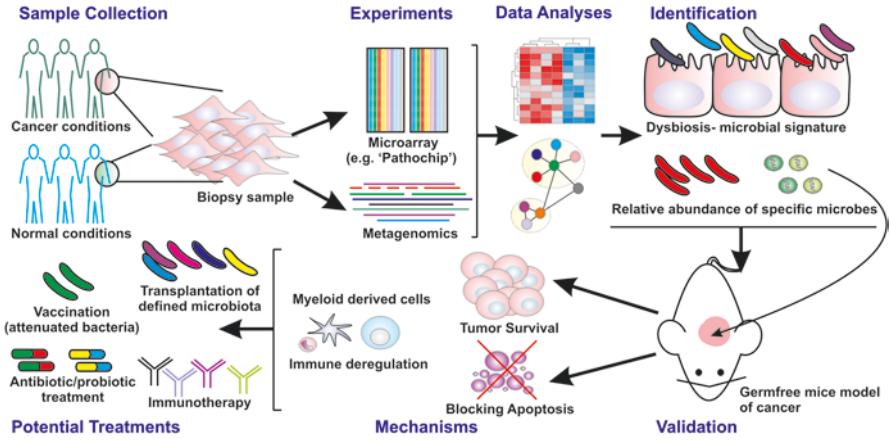

Fig. 1.2 Targeting human microbiota as a potential cancer therapeutic strategy. Through employing high-throughput sequencing (metagenomics) or microarray based technologies it is possible to identify overall microbial composition of disease sample in comparison to normal conditions. Under pathogenic conditions, changes in microbiota composition (dysbiosis) may contribute to cancer development. The 'microbial signature' prevalent in a specific cancer is thus identified and subsequently further investigated in 'germfree' mice model of cancer in order to define the underlying molecular mechanisms. Experiments suggested that microbiota regulates cancer development through blocking immune response and apoptosis, which in turn promotes aberrant cellular proliferation. Treatments targeting microbiota composition, such as antibiotics (to deplete certain bacterial pool), probiotics (to enhance certain microbes), transplantation of defined microbiota (genetically engineered), vaccination using live attenuated bacteria and immunotherapy to regain host immune response have the potential to modulate tumor growth as well as to enhance efficacy of current therapeutic regimen

processing. As a result, this technology has increased sensitivity in comparison to currently available other microbiome screening protocols that involves Next-Gen Sequencing [27]. Furthermore, Next-Gen Sequencing on samples with high microbial load is likely to result in a high degree of selection for the predominant organisms in the sample and therefore selection biases against lower representative organisms. The microarray technology "PathoChIP", although with some limitations in the overall number of organisms, was designed to be inclusive, and so to identify microbial families, which may be represented in the sample [27]. Therefore, this can enrich for organisms that are low representations in the population and thus allow for detection of genomes that are limited in copy numbers. For example, Next-Gen sequencing will have excellent results for acute infections with high copy number of organisms in the gut for example, but may not be as effective for latent infections where few copies of microbial genomes may be present [27]. Using this technology, recently distinct microbial signatures were identified for triple negative breast cancer (TNBC), ovarian carcinoma and oral squamous cell carcinoma [28, 29, 38]. Overall, the identified microbial signatures provide a new paradigm in our current understanding of tumor-associated microbes. However, it is still unclear whether or not these microbes directly contribute to the cancer development or

rather merely exist as commensal microbiota without affecting the cancer microenvironment. Furthermore, the combination of organisms in a population may have additive or synergistic roles in predisposing a tissue to the oncogenic process, or that this combination of organisms has found the perfect niche for their long-term survival. Nevertheless, these microbial signatures provide new diagnostic potential as unique signatures in specific cancers.

To demonstrate the functional importance of the microbiota in cancer development, germfree mice models of cancer were subsequently infected with one or multiple bacteria [39]. However, this 'gnotobiotic model' does not appropriately reproduce the complex composition of the human microbiome. In fact, this experimental approach may either over-emphasize effects due to artificial abundance of a single species or of a group of bacteria, or it may not reveal effects that are due to the requirement of a complex microbial community for the induction of disease by some bacteria. It is therefore imperative to pinpoint the exact environmental conditions that can lead to under-representation and over-representation of certain bacterial species that are associated with cancer, and subsequently to mimic these conditions in experimental models.

1.3 Cancer Associated Microorganisms

To date, the International Agency for Research on Cancer (IARC, http://www.iarc.fr/) categorized 11 infectious microbial agents including seven viruses, three parasites (trematodes), and one bacterium as Group-1 human carcinogens based on their strong association with increasing incidents of several human cancers along with strong evidence from data generated from experiments with laboratory animals (Table 1.2). Although HIV does not directly cause cancer, its infection significantly enhances the occurrence of many tumor viruses (EBV and KSHV) associated human cancers and more recently is also considered an oncovirus, although its effects on the oncogenic process is more indirect. *H. pylori*, HBV, HCV, and HPV together are accountable for more than 90% of all microbes' associated human cancers [56]. The epidemiologic association of some of the human tumor viruses with cancer appeared to be far more complex than what we understood as in general several tumor viruses are highly ubiquitous in nature and found to be associated with more than 95% of the world's population. However, the malignancies that they are associated with are somewhat rare and require specific genetic rearrangements along with number of environmental cofactors that contribute to development of associated cancers. For example, the two gammaherpesviruses—EBV and KSHV are associated with various human neoplasms ranging from epithelial cancers to B-cell lymphomas, particularly in an immune-compromised scenario [57, 58]. EBV is found to be strongly associated with Burkitt's lymphoma (BL), Hodgkin's lymphoma (HL), nasopharyngeal carcinoma (NPC), several form of immunoblastic lymphomas, and to a lesser extent T-and NK-cells lymphoma, gastric and breast carcinomas [9]. KSHV infection causes Kaposi's sarcoma (a rare form of skin

Table 1.2 Group 1 microbial carcinogens[a]

Serial number	Microbial pathogens	Microbial category	Associated cancers	Reference
1	*Helicobacter pylori*	Bacterium	MALT gastric lymphoma, gastric adenocarcinoma	[40, 41]
2	Hepatitis B virus (HBV)	Hepadnavirus	Hepatocellular carcinoma (HCC)	[6, 42]
3	Hepatitis C virus (HCV)	Flavivirus	Hepatocellular carcinoma (HCC)	[6, 43]
4	Human papillomavirus (HPV)	Papillomavirus	Cervical cancer, vaginal cancer, vulva cancer, anal cancer, penile cancer, oropharyngeal carcinoma, head and neck cancer	[7, 44]
5	Epstein-Barr virus (EBV)	Gammaherpesvirus	Nasopharyngeal carcinoma (NPC), Hodgkin's lymphoma (HL), Burkitt's lymphoma, diffuse large B-cell lymphoma (DLBCL) and other immunoblastic lymphomas	[9, 45, 46]
6	Kaposi sarcoma-associated herpesvirus (KSHV)	Gammaherpesvirus	Kaposi's sarcoma (KS), Primary effusion lymphoma (PEL)	[47, 48]
7	Human T-cell lymphotropic virus type 1 (HTLV-1)	Retrovirus	Adult T-cell leukemia/lymphoma (ATL)	[49, 50]
8	Merkel cell polyomavirus (MCPyV)	Polyomavirus	Merkel cell cancer (MCC)	[4, 51]
9	*Schistosoma haematobium*	Trematode	Bladder cancer	[52, 53]
10	*Clonorchis sinensis*	Trematode	Cholangiocarcinoma	[54]
11	*Opisthorchis viverrini*	Trematode	Cholangiocarcinoma	[55]

[a]Designated as per International Agency for Research on Cancer (IARC)

carcinoma) and several other pathologies (such as, Multicentric Castleman's disease or MCD) in immune-suppressive individuals [48]. HBV and HCV are associated with hepatocellular carcinoma (HCC) [59]. HPV, primarily a few high-risk oncogenic strains such as, HPV16 and HPV18, are predominantly associated with several forms of anogenital cancers (cancers of the cervix, anus, penis, vagina and vulva). In addition, HPV is also associated with head and neck cancers, oral cancers and skin cancers [7]. Infection with HTLV-1, the first known human tumor retrovirus, is mostly asymptomatic accountable to approximate 20 million people worldwide. However, in some cases, roughly 3–5% of the infected individuals develop a highly aggressive form of malignancy known as adult T-cell leukemia/lymphoma

(ATL) [60]. MCPyV, the first known human oncogenic polyomavirus, is associated with the majority of cases of Merkel cell carcinoma (MCC), a rare but aggressive form of skin cancer detected in cases of immune-suppressive individuals [[4] and reviewed in [51]].

Interestingly, with the exception of HCV, all human tumor viruses encode at least one oncogene, which was shown to play a direct role in tumor development and progression. However, it has been suggested that many other factors such as inflammation, as well as disruption of the commensal microbiota can also play a role in overall cancer development [13, 61]. For example, even though HPV has a strong transforming ability through exerting E6 and E7 viral oncoproteins mediated activities, vaginal dysbiosis and inflammation around the genital tract due to HPV infection largely contribute to the development of HPV associated anogenital cancers [7]. Hepatitis viruses together with HBV and HCV initially establish a chronic liver infection—a stage known as liver cirrhosis through modulating the host immune response, which eventually develops into HCC, and is accountable to approximately 75% of all clinical observations [6, 59]. The mechanisms by which HBV and HCV promote pathogenesis are distinctly different. Although HBV, but not HCV may directly transform hepatocytes, for both viruses, the pathogenesis of HCC is clearly dependent on immune-related inflammation. While HCV actively evades the initial innate immune response by blocking both type I and type III interferon signaling cascades, the innate immune response to HBV infection is rather weak [62]. However, both viruses are able to compromise the innate as well as adaptive immune responses of the host. Additionally, HBV mediated liver pathogenesis may also connect with gut microbiota particularly the presence of Candida spp., *Saccharomyces cerevisiae,* as well as the less abundance of different varieties of Bifidobacterium spp. Using mice models, the role of the gut microbiota in regulating liver pathology and subsequent development of HCC has been clearly demonstrated as the young mice fail to clear HBV infection until an adult-like gut microbiota is established [63]. It is now clearly understood that inflammation plays a key role in tumor progression associated with all the known tumor viruses. A growing body of evidence clearly suggests that the commensal microbiota along with tumor virus infection are intricately engaged in regulating the immunological response, and thus inflammation which in turn controls cancer propagation, allowing identification of novel molecular targets and their potential for therapeutic interventions [13, 64, 65].

H. pylori infection is considered as the strongest recognized risk factor for the development of gastric adenocarcinoma (non-cardia carcinoma) [66]. Although half of the world's population is infected with *H. pylori*, only a small proportion of individuals develop gastric cancer [67]. In most cases the bacterial infection develops a relatively manageable gastritis, duodenal and stomach ulcers. The worldwide mortality from gastric cancer remains relatively very high, especially in Asia and much of the developing world. *H. pylori* is extremely heterogeneous in nature and is highly adapted for survival in such a hostile condition of gastric mucosa lining contributing a variety of disease pathogenesis. In case of gastric cancer, the major *H. pylori* candidate virulence factors include two cytotoxin encoding genes—cytotoxin-associated gene A (cagA) and vacuolating cytotoxin gene A

(vacA) [68, 69]. Like many other pathogenic and commensal bacteria, *H. pylori* can also profoundly impact the normal functioning of the immune system, of the colonized host. The bacterium activates the TLR4 and TLR2 receptors as well as the NLRP3 inflammasome, thereby promoting the secretion of several interleukins that in turn activate both Th1-cell and regulatory T-cell mediated pro-inflammatory responses [70, 71]. Although *H. pylori* possesses pro-carcinogenic activities and can directly influence gastric mucosa through promoting DNA damage response, development of gastric adenocarcinoma appeared to be much more complicated and involves exposure to the bacterium over several decades, with an initial inflammatory response, epithelium injury and atrophy and a decline in acid secretion function [40, 72–74]. In many developed countries, the occurrence of *H. pylori* infection is decreasing due to better hygiene, recurrent use of broad-spectrum antibiotics and proton pump inhibitors [74]. Interestingly, lowering the incidence of *H. pylori* infection may also result in disruption of the gut microbiota with some unanticipated potential side effects such as, individuals with increased tendency of having asthma, obesity along with elevated risk of development of esophageal and gastric cardiac carcinoma, highlighting the complexity of microbial effects on the development of tissue-specific tumorigenesis [74, 75]. However, this effect may be correlated to a definite genetic predisposition or dietary habits, as the theory was contradicted with the observation, which was found in certain ethnic Malaysian populations known to have a low natural incidence of *H. pylori* infection and generally poor sanitation [76].

With the advent of modern technologies as discussed above, an escalating number of earlier unnoticed pathogens has been discovered, that play critical roles in the development of several human diseases, including cancer. *Fusobacterium nucleatum* (*F. nucleatum*) is such an emerging ubiquitous commensal microbe, usually present in dental plaque, undetected in other parts of the body during normal conditions; however, in disease conditions the bacterium becomes prevalent and disseminates to different body sites. A number of recent studies clearly demonstrated a strong association of *F. nucleatum* with colorectal adenomas and advanced-stage colorectal cancer [23, 77]. For example, *F. nucleatum* introduction to a mouse model of intestinal cancer significantly enhanced the tumor growth through regulation of the NF-κB mediated pro-inflammatory signaling pathway thus affecting the tumor microenvironment [78]. *F. nucleatum* is an adhesive bacterium and encodes several adhesion factors, such as Fap2, RadD, and Aid1 that assist in interspecies interactions in the oral cavity. However, there is only one adhesion molecule, FadA identified, that can bind to the host cells and is one of the best-studied *F. nucleatum* encoded virulence factors [79]. A recent study demonstrated that a host polysaccharide, Gal-Gal-NAc, highly expressed in colorectal carcinoma can be directly recognized by the *F. nucleatum* encoded Fap2 protein, which in turn promotes bacterial attachment [80]. Fap2 also promotes colorectal cancer development by blocking NK-cell mediated immune-surveillance [81]. In addition to the attachment process, FadA can also function as an invasin. FadA inhibits E-cadherin tumor-suppressive activity and consequently, by blocking the interaction of FadA with E-cadherin using a synthetic peptide the host inflammatory response can be abro-

gated, thereby affecting tumor development [79]. Recent studies suggested that *F. nucleatum* increases the ROS production as well as the pro-inflammatory cytokines such as IL-6, IL-8 and TNFα in colorectal cancer [82]. *F. nucleatum* can selectively expand myeloid derived immune cells in colorectal cancer. Myeloid-derived immune cells present in the bone marrow, spleen, or tumor microenvironment can suppress T-cell responses, suggesting a possible mechanism by which *F. nucleatum* modulates the tumor microenvironment and promotes cancer development [83]. In the near future, the detailed elucidation of *F. nucleatum* targeted cellular pathways will provide valuable additional clues for better clinical management of colorectal cancer patients, and their predictive outcomes.

Chronic infections with the liver flukes including *Clonorchis sinensis* (*C. sinensis*), and *Opisthorchis viverrini* (*O. viverrini*) are associated with cholangiocarcinoma [54, 55]. Liver fluke antigens stimulate both inflammatory and hyperplastic changes in the infected bile ducts, which undergo severe pathological transformations. Approximately 5–10% of cholangiocarcinoma is caused by chronic *C. sinensis* infection in endemic areas with low economic status. *Schistosoma haematobium* is a parasitic flatworm associated with bladder cancer that infects millions of people, mostly in the developing world [53]. Research suggest that these helminthes infection are associated with increased cell proliferation, decreased apoptosis, elevation of the anti-apoptotic molecule Bcl-2, down-regulation of the tumor suppressor protein p27, along with increased cell migration and invasion.

1.4 Immune Influence by Microbiota in Promoting Cancer

In recent times, the occurrence of a wide variety of human diseases has been noticeably enhanced, across the globe. The diseases include obesity, asthma, food allergies, inflammatory bowel syndrome, type 1 diabetes and autism, among many others. Ongoing studies have suggested that the disruption and loss of important microbial communities play a major role in the development of such chronic diseases (reviewed in [84]). Loss of such microbial communities have been shown to be associated with changes in living conditions made possible by the introduction of modern life conveniences that has enhanced our daily living standards. For example, extensive use of antibiotics during pregnancy, avoiding breast-feeding and increased rate at which caesarean section is utilized may hamper the horizontal transmission of microbial community from mother to child and in turn result in emergence of several apparently unrelated health problems [84, 85]. An incredible feature of human beings, is not how we respond to pathogenic microorganisms, but more profoundly how we endure the mammoth numbers (estimates of up to three times the total number of host cells) of residing different microbial kingdoms. With the increasing as well as fascinating research in this field, it is now more obvious that the interactions of the early life microbiome with the host are particularly responsible for the commencement of host's immunological tone for the rest of an individual lifespan. Although the most intense effects are focused on the

development of immunity of the gut, microbial communities residing in other areas including skin, mouth and vagina may also contribute to setting the overall immunological, as well as tissue specific immune effects [61, 86, 87].

In addition to emergence of chronic diseases, studies have now clearly demonstrated that microbiota can also influence both cancer propagation and therapeutic response particularly through modulating immune cells and so inflammation. For example, *H. pylori* infection in the gastric mucosa can result in inflammation and aberrant cell proliferation, which subsequently leads to development of stomach cancer [88–90]. On the contrary, a number of intestinal resident bacteria can diminish inflammation, which in turn reduces the rate of cancer cell outgrowth, as well as potentiating the use as cancer immunotherapy. Bifidobacterium can activate dendritic cells in order to present cancer-cell specific antigens to cytotoxic T-cells (CTLs) for killing, which is accompanied by a reduction in growth of subcutaneous melanoma in mice xenograft model. Moreover, introduction of this specific bacterial species in combination with the conventional cancer immunotherapeutic agent "anti-program death-ligand 1 (PD-L1)", can virtually abolish tumor growth [65]. Likewise, combination of *bacteroides thetaiotaomicron* and *B. fragilis* can significantly augment the efficiency of another cancer immunotherapeutic agent 'anti-cytotoxic T-lymphocyte associated protein 4 (CTLA4)' [91]. While *B. fragilis* polysaccharides can enhance anti-tumor immunity, the specific *B. fragilis* polysaccharide A (PSA) promotes an anti-inflammatory state in the intestine [92].

In addition, experiments with pathogen free and antibiotic treated mice demonstrate a typically declined response to CpG oligodeoxynucleotide stimulation in the setting of cancer immunotherapy [13]. The bacterial microbiota also regulates immunity to numerous viral pathogens. It has been demonstrated that previously existing antibodies to enteric bacteria can affect the vaccine responses by cross-reacting with HIV-1 antigens, suggesting a possible mechanistic barrier for proper vaccination [93]. In addition, enteric bacteria can also regulate vaccine responses to influenza in mice through activation of the innate immune receptor, Toll-like receptor 5 (TLR5) [94]. Administration of antibiotics in mice has profound effects on antiviral immunity at another mucosal surface, the lung, since antibiotic treatment prevents normal innate and adaptive immune responses to influenza, causing death of the host [64, 95]. These results emphasize the importance of bacterial microbiota in order to stimulate the antiviral immune responses. However, it is too early for clinicians to decide on using antibiotics as a means of anti-cancer therapy [96]. Expansion of new generation antibiotics targeting individual bacterium along with probiotics [63], as well as introduction of more specific chemotherapeutic agents based on the cancer patient (referred to as 'precision medicine') would definitely change the current scenario (Fig. 1.2).

1.5 Microbes in Cancer Therapy

Owing to the many severe side effects typically associated with conventional chemotherapy, development and inclusion of new anti-cancer therapies are urgently needed. Cumulative studies have resulted in the perception of the microbiota as close associates with their human hosts. Thus, the role of different microorganisms, particularly bacteria and viruses in killing of cancer cells has been explored over extended periods. These studies suggest that these selective microbes should not be harmful to the surrounding non-malignant host cells, and should only replicate in the tumor cells. Furthermore, these microbes should be non-immunogenic and capable of specifically lysing tumor cells [97]. In 1891, an American surgeon William B. Coley observed that administration of certain heat-killed microbes which included *Streptococcus pyogenes* and *Serratia marcescens* (referred to as 'Coley's toxin') can radically cause tumor regression [98]. Therefore, the use of Coley's toxin was often determined as an alternative strategy for the successful treatment of various forms of cancer for which no alternative treatments were available [98]. However, in many cases treatment regimens with Coley's toxin resulted in a number of side effects. This led to limited enthusiasm for this treatment, and is not generally accepted among clinicians. The most promising clinical application of microbial agents in the treatment of cancer was first described in 1976, when a urinary bladder cancer patient was treated by the introduction of the Bacillus Calmette-Guérin (BCG) vaccine, a live attenuated strain of *Mycobacterium bovis, and a* standard vaccine protocol against tuberculosis (TB) infection [99, 100]. Currently, this method is considered as one of the most successful immunotherapy against superficial urinary bladder cancer. In addition, BCG mediated immunotherapy has also been investigated in case of colorectal carcinoma [100]. The anti-cancer effect of BCG is based on the induction of a local immune response and the production of cytokines such as IL-2, TNF-α and INF-γ. However, the BCG vaccine has also shown multiple side effects and incompetence in approximately 50% of the treated patients [100, 101]. Similar to the BCG vaccine, *Lactobacillus* species have also shown promising outcomes in regards to the recurrence of urinary bladder cancer [101]. A number of bacterial species under *Bifidobacterium* genus, including *B. longum*, *B. infantis* and *B. adolescentis* appear to possess potential anti-cancerous agents in mice models [102, 103]. Likewise, several *Clostridium* species such as *C. histolyticum, C. perfringens* and *C. novyi* can also block tumor growth in animal models [104, 105]. Both in case of *Bifidobacterium* and *Clostridium* species, the anti-tumorigenic effects were determined using animal models; lack of patient data and significant associated toxicities raise uncertainties in their therapeutic capacity. Administration of live attenuated *Salmonella enteric* also causes tumor regression in mice models [106]. Subsequently, a genetically modified *Salmonella* strain 'VNP20009' was generated and is being currently tested for the treatment of various cancers in Phase I clinical trial [107]. Later, a number of other strains of

Salmonella species have been generated and demonstrated potential tumor regression activities in various cancer types [108, 109].. Interestingly, natural tumor regression can also occur in the presence of a number of other bacterial infections including Diphtheria, Gonorrhoea, Syphilis and Tuberculosis, and viral pathogenesis such as hepatitis, influenza, rubella and smallpox [110]. In addition, a number of bacterial toxins and metabolites can significantly influence tumor growth both in experimental models and in clinical settings (Table 1.3). For example, while 'azurin', a peptide encoded by *Pseudomonas aeruginosa,* induces apoptosis and

Table 1.3 Microbial agents as anticancer therapy

Anticancer agents	Microorganisms	Mechanism of action	Reference
Azurin	*Pseudomonas aeruginosa*	Deregulates cell proliferation, induces caspase-dependent apoptosis, and blocks angiogenesis	[111]
Exotoxin A	*Pseudomonas aeruginosa*	Inhibits protein synthesis by inducing ADP-ribosylation of cytoplasmic elongation factor 2	[120]
Diphtheria toxin	*Corynebacterium diphtheriae*	Inhibits protein synthesis by inducing ADP-ribosylation of cytoplasmic elongation factor 2, increases apoptosis	[112]
Actinomycin D	*Streptomyces* spp.	Inhibits transcription through blocking RNA polymerase activity	[116]
Bleomycin	*Streptomyces verticillus*	Inhibits DNA synthesis. However, the exact mechanism is not yet known	[114]
Daunomycin	*Streptomyces coeruleorubidus*	Interacts with DNA by intercalation and thereby inhibits macromolecular biosynthesis. It also inhibits the progression of topoisomerase II	[121]
Doxorubicin	*Streptomyces pneuceticus*	Interacts with DNA by intercalation and thereby inhibits macromolecular biosynthesis. It also inhibits the progression of topoisomerase II	[118]
Epirubicin	*Streptomyces pneuceticus*	Forms strong complex with DNA by intercalation between base pairs and also inhibits topoisomerase II activity	[113]
Idarubicin	*Streptomyces pneuceticus*	Forms strong complex with DNA by intercalation between base pairs and also inhibits topoisomerase II activity	[115]
Mitomycin C	*Streptomyces caespitosus*	Inhibits cell proliferation through alkylation of DNA	[117]
Geldanamycin	*Streptomyces hygroscopicus*	Inhibits telomerase assembly through disrupting HSP90-telomerase complex; inhibits src tyrosine kinase activity	[122, 123]
Rapamycin	*Streptomyces hygroscopicus*	Induces autophagy through blocking mTOR pathway	[124]
Wortmannin	*Talaromyces wortmanni*	Blocks autophagy through inhibiting phosphatidylinositol 3 (PI-3) kinase	[125, 126]

blocks angiogenesis, 'endotoxinA' encoded by *Pseudomonas aeruginosa,* and 'diphtheria toxin' encoded by *Corynebacterium diphtheriae* inhibit protein synthesis by inducing ADP-ribosylation of cytoplasmic elongation factor 2 [111, 112]. Interestingly, several species under *Streptomyces* genus produce a number of metabolites (actinomycin D, bleomycin, doxorubicin, epirubicin, idarubicin, and mitomycin C), that act as potential DNA damaging anti-cancer agents at least in laboratory experimental settings [113–118]. However, bacteria and viruses are not the only agents that can induce tumor regression. Additional evidence has shown that a number of protozoa, such as *Toxoplasma gondii* and *Besnoitia jellisoni* can also activate macrophages and thereby causing tumor regression [119]. Although microbial treatment of cancer is providing new perspective, the use of microorganisms to target tumors has certain limitations. For example, the biosafety, genetic instability and the confounding interactions of the microorganisms with chemotherapeutic agents should also be considered in greater detail.

1.6 Conclusion and Future Perspectives

In this chapter, we highlight the recent advances in understanding the human microbiome and its intricate association with cancer, as well as promising future avenues of research, including the identification of novel molecular targets for therapeutic enhancement, development of vaccines and cancer prognostic markers (Fig. 1.2). The host and the microbiome continuously interact with each other, and are considered to be two fundamental constituents of the 'holobiome', resulting in maintenance of a healthy steady state of cellular homeostasis. However, alterations of the host-microbiome interactions coupled with germ-line encoded disease susceptibility risks, resulted in onset of several disorders, including cancer. The advent of high-throughput technologies has radically changed our understanding of the host microbiome and its ability to play a major role in cancer development. However, extensive research will be necessary to delineate the roles of organ-specific microbiome in cancer development. The effects of one microbiome on tumor progression in other distal locations, and alterations in immune functions by the microbiota, as well as the potential involvement of other commensal microbial kingdoms, such as fungi, archaea and parasites, along with environmental factors (such as food habit, smoking) in cancer biology needs to be further explored. As the scientific community continues to generate more microbiome data, and integrate other "omics" types such as transcriptomics, proteomics, and metabolomics from well-phenotyped cohorts, we would be able to discover novel microbial signatures that are associated with disease onset and progression in many diseases, including cancer. These microbiome signatures along with circulating metabolites have the potential to be utilized in diagnostics and therapeutics strategies.

Overall, the outlook is optimistic, but there are also substantial challenges in the field. To implement microbiome-based diagnostics and therapeutics, we need to develop uniform collection, sequencing, and analysis standards that would

enhance reproducibility of results across centers and reduce biases in their interpretation. In general, the recent investigations are based on identification of microbes associated with different cancers. However, the trend should be towards better defining the underlying mechanisms by which microbiota manipulate cancer microenvironment along with development of appropriate biomarkers. Once the most favourable microbial composition for each clinical condition has been identified, the next challenge will be how to modify the patient's microbiota in order to enhance cancer therapy. In addition, we are only beginning to appreciate the contribution of other microbial kingdoms such as fungi, bacteriophages, and parasites as well as the transkingdom interactions along with host cellular signalling pathways. As we unravel aspects of these intricate interactions, we will begin to understand the influence of the microbiome with both positive as well as negative regulatory impacts, on the host in connection with development of various pathophysiological conditions, such as cancer. Although the field of therapeutic intervention through targeting the microbiota is still in its infancy, a number of approaches has already been made. For example, the validation of the microbiota as a therapeutic target is provided by studies showing that patients can be recolonized with a resilient and stable modified microbiota to fight antibiotic resistant pathogens. The ultimate goal is to discover a bacterial species or a combination of species that both reduces systemic toxicity and promotes anticancer therapy. Thus, targeting the microbiota in cancer and other diseases is likely to become one of the next frontiers for precision and personalized medicine.

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Chapter 2
Infection Based Gastric Cancer

Lydia E. Wroblewski and Richard M. Peek Jr.

Abstract Gastric cancer is one of the leading causes of cancer-related death in the world. *Helicobacter pylori* is currently the strongest known risk factor for this disease and is classified as a type I carcinogen by the World Health Organization. Many factors play a role in the progression towards gastric cancer including, but not limited to, bacterial virulence factors, host genetics, diet, and the gastric microbiota. The stomach, once thought to be a sterile environment, is now known to host a rich microbiota, which is unique to each individual. A complex interplay exists between *H. pylori* and the gastric microbiota which may one day become a target for personalized medicine to attenuate the progression towards gastric cancer. In this chapter, we discuss how the infectious bacterium, *H. pylori*, interacts with its host to augment the risk of developing gastric cancer.

Keywords *Helicobacter pylori* · Gastric cancer · Microbiota · Infectious agent

2.1 Infection-Associated Cancers

Infectious agents are major contributors to the development of human cancer and collectively they impose a large burden on global health. In 2008, two million of an estimated 12.7 million new cases of cancer were ascribed to infections. Perhaps not surprisingly, 80% of these infection-based cancers occurred in less developed regions of the world, which is likely attributable to a inadequate preventative treatment of infectious agents [1].

Francis Peyton Rous first noted the association between infection with specific pathogens and cancer over a century ago in 1911 when he demonstrated that a malignant tumor (a sarcoma in chickens) was transmissible. This is now known as the Rous sarcoma virus and its pathogenesis is still widely studied over 100 years from its discovery [2]. In 2012, the International Agency for Research on Cancer

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Table 2.1 Group 1 infectious agents and the major cancer sites they are associated with

Cancer site	Infectious agent
Stomach	*H. pylori*, EBV
Liver	HBC, HCV, *Opisthorchis viverrini*, *Clonorchis sinensis*
Cervix	HPV
Anogenital	HPV
Nasopharynx	EBV
Oropharynx	HPV
Kaposi's sarcoma	Human herpes virus type 8
Non-Hodgkin lymphoma	*H. pylori*, EBV, HCV, human T-cell lymphotropic virus type 1
Hodgkin's lymphoma	EBV
Bladder	*Schistosoma haematobium*

(IARC) classified eleven infectious agents as harboring carcinogenic potential for humans [1, 3]. These include *H. pylori*, hepatitis B virus (HBV), hepatitis C virus (HCV), *Opisthorchis viverrini*, *Clonorchis sinensis*, human papillomavirus (HPV), Epstein-Barr virus (EBV), human T-cell lymphotropic virus type 1 (HTLV-1), Merkel Cell polyoma virus (MCPv), human herpes virus type 8 (HHV-8; also known as Kaposi's sarcoma herpes virus KSHV), and *Schistosoma haematobium*. The cancers these infectious agents are associated with include, but are not limited to, gastric, liver, cervical and bladder, and are summarized in Table 2.1.

One of the primary infectious agents deemed a class I carcinogen is *H. pylori*. This single bacterium accounts for a staggering 32.5% of the two million new cancer cases attributable to infections worldwide occurring in 2008 [1]. To date, *H. pylori* is the only bacterium that is recognized as causally being associated with malignant neoplasia in humans and it confers an attributable risk of approximately 89% for non-cardia gastric carcinoma which translates to around 780,000 new gastric cancer cases, emphasizing the role of *H. pylori* as a major cause of cancer [4].

2.2 Gastric Cancer

Gastric cancer was the leading cause of cancer-related death in the developed world until the mid-1930s and despite a significant decrease in incidence rates, gastric cancer is still the third leading cause of cancer-related death in the world, resulting in close to 740,000 deaths in 2008. Within the United States the 5-year survival rate is surprisingly low, at less than 15% [1, 5–7]; such high mortality rates are primarily thought to be due to late-stage detection.

The incidence and mortality rates of gastric adenocarcinoma in developed countries have declined significantly over the past century. This is primarily attributed to a decline in intestinal-type adenocarcinomas in the distal stomach and may be related to decreased transmission of *H. pylori* in childhood following improved

hygiene and smaller family units and/or changes in food preservation and storage [6, 8, 9]. Distal gastric adenocarcinomas are strongly associated with *H. pylori* infection, but the causal relationship between *H. pylori* and gastric cardia adenocarcinomas is less well defined. Conversely, the incidence rates of cancers localized to the cardia, as well as Barrett's esophagus and adenocarcinomas originating in the gastroesophageal junction, have been increasing in both the United States and Europe. This increase is seen predominately in white males and to date the reasons for this increase are unclear [9–11].

The Cancer Genome Atlas (TCGA) research network proposed a new molecular classification whereby gastric cancer is divided into four subtypes and EBV-associated gastric tumors have been classified as a newly distinct subtype of gastric cancer; EBV-positive tumors [12]. The three other subtypes of gastric cancer are termed microsatellite-instable tumors, genomically stable tumors, and tumors with chromosomal instability. EBV-positive tumors contain *PIK3CA* mutations, DNA hypermethylation, and increased expression of *JAK2*, *CD274*, and *PDCD1LG2* [12].

Adenocarcinoma is the most common type of cancer affecting the stomach, but lymphoma and leiomyosarcoma may also occur. Distinct variants of gastric adenocarcinoma can be separated into two types which may be differentiated histologically; intestinal-type adenocarcinoma, which progresses through a series of well-defined histological steps and diffuse-type gastric cancer, which consists of individually infiltrating neoplastic cells that do not form glandular structures [13].

The strongest identified risk factor for developing gastric adenocarcinoma is chronic infection with *H. pylori* and whilst most human gastric cancers arise following long-term infection with *H. pylori*, emerging data suggest that other components of the gastric microbiota may also influence gastric disease progression (see Sect. 2.3.5). The reported degree to which *H. pylori* increases the risk for gastric adenocarcinoma varies between studies and is likely dependent on several factors including patient age, selection of controls, and the site and stage of gastric cancer. In one study, infection with *H. pylori* was associated with 6.2% of all gastric cancers [4]. In another study, the combined incidence of intestinal and diffuse-type gastric cancer in *H. pylori*-infected individuals was reported to be approximately 3%, compared with 0% in uninfected persons [14]. As our knowledge currently stands, it is not possible to predict which infected individuals will develop gastric cancer and what form this will take.

2.3 Factors That Influence Gastric Carcinogenesis

2.3.1 Host Genetics

The combination of a more virulent strain of *H. pylori* infecting genetically susceptible hosts further increases the risk of developing gastric cancer. For example, infection with *H. pylori* increases gastric mucosal expression of the pro-inflammatory

cytokine, IL-1ß. Individuals who possess polymorphisms in IL-1ß that culminate in high expression levels are at a significantly higher risk of developing distal gastric adenocarcinoma compared to individuals with genotypes that limit IL-1ß expression, but only when colonized with *H. pylori* [15]. Further, the combination of colonization with *H. pylori cagA*$^+$ or *vacA* s1-type strains (discussed further in *H. pylori* section 2.3.3) in conjunction with high-expressing IL-1ß polymorphisms on the host side, confers a 25- or 87-fold increase in risk, respectively, for developing gastric cancer compared to uninfected individuals [16]. Polymorphisms that increase expression of the pro-inflammatory cytokines TNF-α and Il-10 are also associated with an augmentation in risk of developing gastric cancer and its precursors in the presence of *H. pylori* [17].

2.3.2 The Environment

Case-control studies have identified clear associations between diet and the risk of developing gastric cancer. Diets rich in fruits and vegetables and therefore antioxidants are protective against gastric cancer. Conversely, diets containing a high amount of salted, pickled, smoked or poorly preserved foods, diets rich in meat which induces production of nitrosamines, and those with low fruit and vegetable content are most commonly associated with an increased risk for developing gastric cancer [18–24].

When *H. pylori* is present, high dietary salt intake and low iron levels are highly associated with an increased risk for developing gastric cancer [25–27]. In animal models, high salt diets have been reported to increase expression of the *H. pylori* virulence factors CagA, VacA and UreA [28–30]. Similarly, iron deficiency in *H. pylori*-infected persons is also thought to accelerate the development of carcinogenesis by increasing the virulence potential of *H. pylori* [26].

2.3.3 Infectious Agents

2.3.3.1 *H. pylori*

H. pylori is a epsilonproteobacterium and a member of the *Helicobacteraceae* family that selectively colonizes gastric epithelium. *H. pylori* has colonized humans for around 60,000 years; infection is usually acquired in childhood and in the absence of combined antibiotic therapy, can persist for the life time of the host [31]. This long standing relationship between *H. pylori* and its human host, combined with approximately half of the world's population currently being colonized with *H. pylori* has driven many investigators to try and define specific mechanisms through which *H. pylori* interacts with humans and induces disease [32].

2.3.3.2 *H. pylori* Virulence Factors

H. pylori virulence factors play a key role in determining the risk of developing gastric cancer. One *H. pylori* pathogenic constituent that is linked to carcinogenicity is the *cag* pathogenicity island (*cag*PAI), which contains a cluster of genes encoding proteins that form a type IV bacterial secretion system (T4SS). The *cag* T4SS translocates CagA from adherent *H. pylori* across the bacterial and epithelial membranes into host cells. Around 60% of *H. pylori* isolates from Western countries contain the *cag*PAI and almost all strains from East Asia are positive for *cag*PAI [33–36]. Infection with *cagA*-positive *H. pylori* strains has been associated with developing intestinal and diffuse gastric adenocarcinoma at 2–3 times the frequency of those infected with *H. pylori* strains that are *cagA*-negative [37, 38].

CagA exists in alternative structures and contains different glutamate-proline-isoleucine-tyrosine-alanine (EPIYA) motifs, which may also be used as indicators of pathologic outcome [39–41]. Four different EPIYA motifs (EPIYA-A, -B, -C, or -D) have been identified [39–41]. EPIYA-A and EPIYA-B motifs are found in most strains, while the EPIYA-C motif is predominately found in Western strains and the number of EPIYA-C sites is associated with an elevated risk of developing gastric cancer [42]. Strains that contain the EPIYA-D motif are typically East Asian strains and are associated with increased pathogenesis compared with strains harboring C-type CagA motifs (Fig. 2.1) [39, 43]. Following translocation, CagA is tyrosine

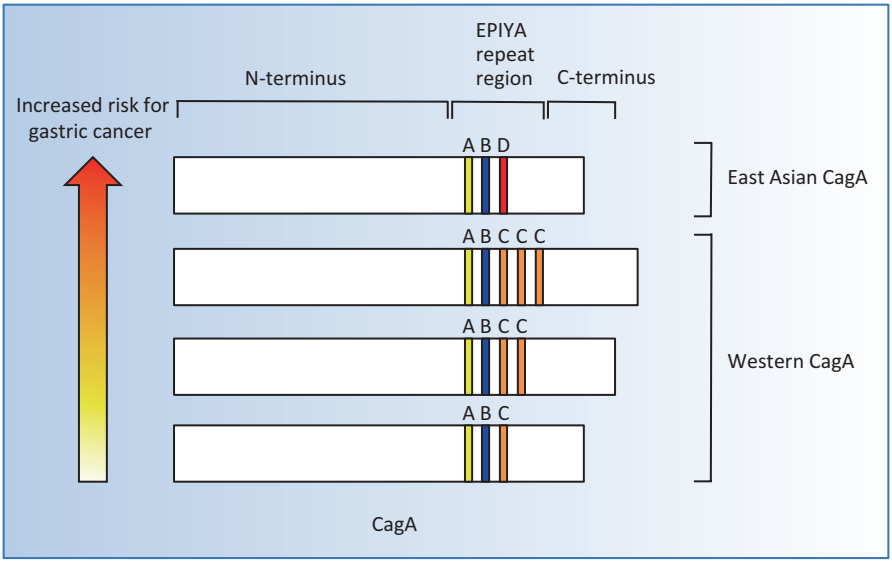

Fig. 2.1 Schematic representation of CagA EPIYA motifs. EPIYA motifs are sites of tyrosine phosphorylation. EPIYA-D motifs are commonly found in East Asian CagA sequences, EPIYA-C motifs are generally found in Western CagA sequences and EPIYA-A and EPIYA-B motifs are found in most strains. EPIYA motifs can be used to predict pathologic outcome, with EPIYA-D motifs associated with increased pathogenesis compared to a single EPIYA-C motif

phosphorylated at EPIYA motifs and can induce cellular response with carcinogenic potential. Non-phosphorylated CagA also exerts effects within host cells that contribute to pathogenesis. Unmodified CagA targets many cellular effectors including apical-junctional components, the hepatocyte growth factor receptor c-Met, the phospholipase PLC-γ, the adaptor protein Grb2, and the kinase PAR1b/MARK2, leading to pro-inflammatory and mitogenic responses, disruption of cell-cell junctions, and loss of cellular polarity [44–51]. Independent of CagA, *H. pylori* can also induce mislocalization of the tight junction proteins occludin and claudin-7 and alter barrier function [52, 53].

Another widely studied *H. pylori* virulence factor is the multifunctional cytotoxin VacA which causes vacuolation, altered plasma and mitochondrial membrane permeability, autophagy, and apoptosis [54, 55]. The *vacA* gene is found in all strains of *H. pylori*, and contains a number of variable loci in the 5′ region of the gene termed s, i and m regions. This 5′ terminus encodes the signal sequence and amino-terminus of the secreted toxin (allele types s1a, s1b, s1c, or s2), an intermediate region (allele types i1 or i2), and a mid-region (allele types m1 or m2) [56, 57]. Strains containing type s1, i1, or m1 alleles are highly associated with gastric cancer [56, 58, 59] and are associated with a greater risk of developing gastric cancer than *cag* status [57, 60, 61]. VacA and CagA may also counter-regulate each other's actions to manipulate host cell responses [62–64].

Blood group antigen binding adhesin (BabA) and Sialic acid-binding adhesin (SabA) are two other important *H. pylori* constituents that have been linked to the development of gastric cancer [65]. BabA is an outer membrane protein that binds to fucosylated Lewis^b antigen (Le^b) on the surface of gastric epithelial cells [65–68]. The presence of *babA2,* the gene encoding BabA*,* is associated with gastric cancer [65], and BabA expression is linked with adenocarcinoma of the gastric cardia [69]. The combined effect of BabA with *cagA* and *vacA* s1 alleles is strongly linked to a more severe gastric disease outcome [65, 70]. Sialyl-Lewis^x is expressed in the gastric epithelium and expression is increased by chronic inflammation [71]. SabA binds to sialyl-Lewis^x antigen, suggesting that *H. pylori* may modulate sialyl-Lewis^x in the host to enhance attachment and colonization [72].

2.3.4 Epstein-Barr Virus (EBV)

EBV infection is another pathogen that is associated with gastric cancers. EBV-positive tumors comprise almost 10% of gastric cancers, are associated with extensive gene methylation, predominately affect males, and tumors are generally located in the cardia or corpus, and are less frequently found in the antrum [73, 74]. EBV and *H. pylori* may act synergistically in the gastric epithelium to promote the progression towards gastric cancer and the majority of EBV-positive individuals are also co-positive for *H. pylori* [75]. A case-control study has shown that the combination of EBV and *H. pylori* induces severe inflammation and, in this way, augments the risk of developing intestinal type gastric cancer [76]. A meta-analysis

with meta-regression to control for heterogeneity across studies also supported the notion that infection with EBV increases the risk of developing gastric cancer [77]. In a recent mechanistic study, EBV was shown to methylate the phosphatase SHP1 and thereby prevent SHP1 from dephosphorylating CagA. This perturbation increases the oncogenic activity of CagA and may increase the synergistic effect of EBV and *H. pylori* [78].

It has been shown that patients who present with the highest levels of antibodies against EBV and *H. pylori* also express the highest levels of immune cell infiltration, and are therefore, at increased risk for developing more severe inflammation. In a recent cross-sectional study of 127 patients with gastric cancer, the presence of elevated serum levels of the cytokine interferon-gamma (IFN-γ) has been associated with EBV reactivation and intestinal gastric cancer. However, IFN-γ can exert both pro-inflammatory and anti-inflammatory effects, and further studies need to be conducted to determine if IFN-γ is acting to repress EBV activity or is augmenting EBV and *H. pylori*-induced gastric cancer progression [79].

2.3.5 The Human Gastric Microbiome

The gut microbiota is essential to maintain host physiology through its integral role in cellular metabolism, nutrient absorption and immune defense against invading pathogens. When the microbiota is altered, homeostasis is also disrupted, and diseases may develop. Historically, research has focused on a single organism causing disease, for example *H. pylori* and gastric cancer; however, a rapid burst in molecular technologies such as next-generation sequencing in combination with computational analysis and new and well-designed animal models have transformed our understanding of how the microbiota is associated with disease states. A diverse bacterial community is found within the stomach with colonization densities reported to range from between 10^1 and 10^3 colony forming units/g [80]. Emerging data strongly suggest that the gastric microbiota affects gastric homeostasis in combination with *H. pylori* infection [81].

The gastric microbiota in *H. pylori*-negative individuals is highly diverse. Through one sequencing study, 128 phylotypes were identified within eight bacterial phyla; and the five most abundant phyla were Proteobacteria, Firmicutes, Bacteroidetes, Fusobacteria, and Actinobacteria [82, 83]. In an independent study using tagged 454 pyrosequencing analysis, 262 phylotypes representing 13 phyla were identified in gastric biopsies from *H. pylori*-negative persons [84]. Even though the results of the analysis vary depending on the sequencing approach and sample preparation, in addition to the large variability between the microbiota in different individuals, it is clear that the gastric microbiota is highly diverse [82, 84]. In stark contrast, in *H. pylori* infected individuals, *H. pylori* was found to be the single most abundant phylotype present in the stomach and accounts for between 72% and 97% of all sequence reads [82, 84, 85].

Currently there are very few studies that have examined differences in microbial composition and outcomes stratified by disease. Atrophic gastritis is a key step in the histologic progression to intestinal-type gastric cancer and predisposes the stomach to elevated pH [13]. The hypochlorhydric environment found in atrophic gastritis permits colonization of other bacteria that may enter the stomach and may further promote the progression towards gastric cancer. In one study, the microbiota of patients with gastric cancer was found to be equally as complex as the microbiota of dysplastic patients with five predominant bacterial phyla identified in both groups; Firmicutes, Bacteroidetes, Proteobacteria, Actinobacteria, and Fusobacteria. *H. pylori* was detected in relatively low abundance and the microbiota was instead dominated by species of *Streptococcus*, *Lactobacillus*, *Veillonella*, and *Prevotella* [86]. A more recent study using pyrosequencing found distinct differences when the gastric microbiota was compared in different disease stages from chronic gastritis, to intestinal metaplasia and gastric cancer. In gastric cancer, the Bacilli class and Streptococcaceae family were significantly increased compared to what was found in chronic gastritis and intestinal metaplasia, where the Epsilonproteobacteria class and Helicobacteraceae family were both decreased [87]. In a recent large study, the gastric microbiota was compared in chronic gastritis and gastric cancer and significant differences were identified between the two groups. Specifically, the microbiota in gastric cancer had decreased diversity, reduced *Helicobacter* abundance and over-abundance of *Citrobacter*, *Clostridium*, *Lactobacillus*, *Achromobacter* and *Rhodococcus*, which are usually found in the intestinal microbiota [88].

These studies are intriguing and demonstrate associations between the human gastric microbiota and *H. pylori* with gastric disease, however, they are not able to differentiate between cause and effect. To start to address whether changes in the gastric microbiota play a direct role in the development of gastric cancer, or are secondary to the changing gastric environment, further detailed molecular studies to define the composition of the gastric microbiota in well-characterized human populations, with and without gastric cancer will need to be conducted. As of now, infection with *H. pylori* is the strongest known risk factor for developing gastric cancer, however, a large longitudinal human study suggests that other components of the gastric microbiota may influence gastric disease progression. In a 15-year follow-up study of 3365 subjects, antibiotic treatment of *H. pylori* infection significantly reduced the incidence of gastric cancer despite less than half of the treated individuals remaining free of *H. pylori* infection. The incidence of gastric cancer was decreased to a similar level in individuals that remained free of *H. pylori* over 15 years versus those where eradication was not successful, suggesting that treatment with antibiotics may modify the microbiota in such a way that the development of gastric cancer is attenuated despite the presence of *H. pylori* [89]. Along similar lines, computational analysis of bacterial DNA within known cancer genomes determined that gastric adenocarcinoma contained the second highest number of bacterial DNA sequences. Interestingly, this bacterial DNA was not *H. pylori*, but was instead, *Pseudomonas* [90].

2.3.6 The Rodent Gastric Microbiome

Animal models greatly increase our ability to establish causality. Inbred mice with defined genotypes are frequently used as a model of gastric carcinogenesis and transgenic mice can be generated to allow for in-depth analyses of host responses.

Similar to in the human stomach, the phylotypes with the most members in the mouse gastric environment are *Bacteroidetes*, *Firmicutes*, *Proteobacteria*, and *Actinobacteria* [91]. Similar to in humans, *H. pylori* induces chronic atrophic gastritis in the mouse gastric mucosa; however, *Acinetobacter lwoffii* in the absence of *H. pylori* can also induce gastric inflammation and metaplastic changes comparable to that induced by *H. pylori* [92]. Also, the extent to which inflammation is induced by *H. pylori* can vary depending on the composition of the mouse gastric microbiota with different ratios of *Lactobacillus* species ASF360 and ASF361 altering the outcome for the inflammation and injury responses when mice were subsequently challenged with *H. pylori* [91].

Gnotobiotic mice provide a powerful model in which the microbiota can be carefully controlled by incremental addition of individual or collections of microorganisms. INS-GAS mice are transgenic hypergastrinemic mice that, in the presence of a complex gastric microbiota, spontaneously develop gastric cancer [93, 94]. However, development of gastric cancer was delayed by over a year in gnotobiotic INS-GAS mice [95]. In the context of *H. pylori* infection, gnotobiotic mice challenged with *H. pylori* developed less severe lesions and were slower to develop gastric cancer than *H. pylori*-infected INS-GAS mice with a complex microbiota [95]. Subsequent work has shown that a microbiota containing only three species of commensal bacteria (ASF356 *Clostridium* species, ASF361 *Lactobacillus murinus* and ASF519 *Bacteroides species*) was sufficient to promote gastric cancer in *H. pylori*-infected INS-GAS mice to the same extent as what was seen in *H. pylori*-infected INS-GAS mice with a complex microbiota [96].

Extragastric constituents of the microbiota may also influence outcomes of *H. pylori*-induced gastric cancer in mice. Co-infection of mice with the intestinal *Helicobacter* species *H. bilis* or *H. muridarum* significantly decreased *H. pylori*-induced gastric disease by altering T helper 1-type cell responses [97, 98]. However, pre-existing infection with *H. hepaticus* increased *H. pylori*-induced gastric disease through a T helper 17-type cell response to the combined infection [97]. Helminth infections may also decrease the degree to which *H. pylori*-induces changes in the microbiota of mice [99].

Although great advances are being made in understanding the complex interplay between the microbiota and *H. pylori* in the development of gastric cancer in animal models, rodent models have several limitations. Among other problems, rodents are not naturally infected with *H. pylori* and need to be experimentally infected with rodent adapted strains. Also, the topography of *H. pylori* colonization in rodent stomachs does not precisely reflect that of humans [81]. An exciting animal model for investigating interactions between *H. pylori* and the gastric microbiota is the rhesus monkey (*Macaca mulatta*). Rhesus monkeys are naturally infected early in

life with *H. pylori* strains that are indistinguishable from human strains. In addition, the rhesus monkey stomach is similar to humans, in contrast to rodents, which possess a forestomach, and gastric biopsies can be obtained over time by endoscopy [100]. Similar to humans, *Helicobacter* species formed the majority of the gastric microbiota when present in rhesus macaques [100].

2.4 Conclusions

Gastric cancer culminates in a high number of cancer-related deaths throughout the world and understanding the complex interplay between host factors, *H. pylori,* and the gastric microbiota will be critical to identify individuals who are most at risk of developing gastric cancer (Fig. 2.2). There has been some success in generating a *H. pylori* vaccine in *H. pylori* naive children [101], but eradication of *H. pylori* using antibiotics is not always successful and contributes to the global problem of bacterial resistance. Moreover, there is mounting evidence to suggest *H. pylori* may be beneficial to a large proportion of infected individuals who may be protected against esophageal diseases, gastric reflux disease and some allergic and autoimmune diseases. Thus, it is increasingly important to identify the 1–3% of individuals colonized by *H. pylori* that will develop gastric cancer and specifically test and treat these persons.

In the future, treatment for gastric cancer may soon involve personalized medicine targeting elements such as the gastric microbiota. Indeed, pioneering work

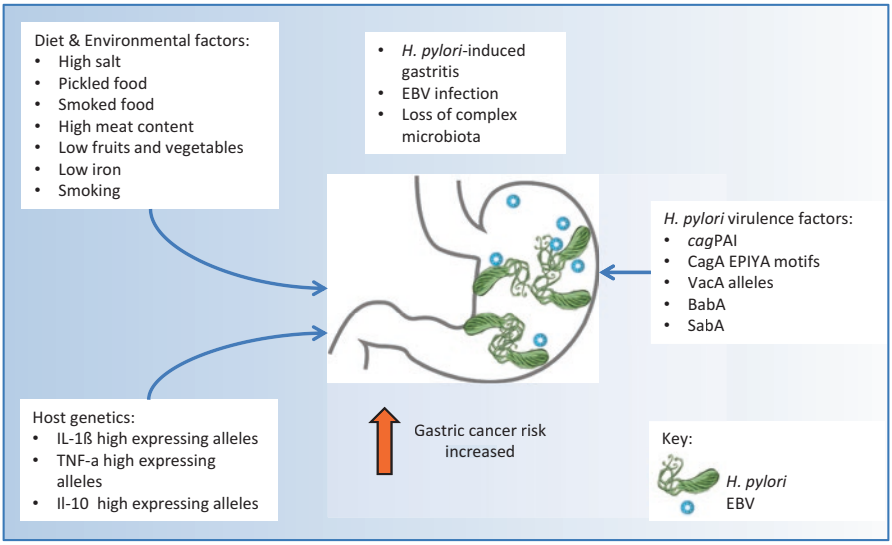

Fig. 2.2 Schematic representation of gastric cancer risk factors in combination with *H. pylori*-induced chronic gastritis

recently published has demonstrated that cancer patients have a better therapeutic outcome with PD-1 inhibitor immunotherapy when their gut microbiome is complex and intact compared to individuals who had received antibiotics that disrupted the microbiome around the time of receiving immunotherapy [102]. The hope is that we may be able to identify groups of bacterial taxa present in the stomach that are predictive of gastric disease outcome. It may also be possible to manipulate an individual's specific microbiota to produce more favorable outcomes following infection with *H. pylori*. Exploiting the microbiome to improve gastric cancer outcomes will be challenging given the large amount of variation between individuals and detailed analyses of the human gastric microbiome still need to be completed. Furthermore, it will be critical to determine cause and effect outcomes when targeting the gastric microbiome to alter disease outcome [103]. Ultimately, understanding the dynamics of the microbiota, along with host genetic and dietary factors, and *H. pylori* virulence factors will be essential to devise a plan to treat patients with precancerous gastric disease.

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Chapter 3
Role of Infectious Agents on Development of Esophageal Carcinomas

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Abstract Comprising two primary histological subtypes, esophageal squamous cell carcinoma and esophageal adenocarcinoma, esophageal cancer remains among the most aggressive forms of human malignancy. Despite advances in our understanding of the genetic landscape of esophageal cancer, patient outcomes remain poor, suggesting that cell extrinsic factors may influence disease pathogenesis. Interest in defining roles for infectious agents in esophageal carcinogenesis is rapidly emerging as an increasing number of clinical studies have linked various pathogens with esophageal cancer. Here, we review the current literature characterizing bacterial, viral, fungal and parasitic pathogens in the esophagus in the context of homeostasis and carcinogenesis. We discuss global changes in the microbial composition of the esophagus and adjacent organs as they relate to esophageal cancer. We further provide a comprehensive overview of the relationship between specific pathogens, including *Helicobacter pylori*, *Herpesviridae* and Human Immunodeficiency Virus, and esophageal cancer.

Keywords Barrett's esophagus · Esophageal adenocarcinoma · Esophageal squamous cell carcinoma · Microbiome · *H. pylori* · Helicobacter pylori · Human papilloma viruses · Epstein-Barr virus · Herpes simplex virus · Cytomegalovirus · Varicella-zoster virus · Human immunodeficiency virus · Candida · Chronic mucocutaneous candidiasis · Trypanosoma cruzi

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3.1 Introduction

Esophageal cancer is the eight most prevalent cancer type and the sixth-leading cause of cancer-associated mortality worldwide [1, 2]. Esophageal adenocarcinoma (EAC) and esophageal squamous cell carcinoma (ESCC) comprise the two primary histological subtypes of esophageal malignancy, each with distinct epidemiology and pathophysiology. In addition, non-epithelial tumors such as lymphoma and malignant melanoma arise in the esophagus, albeit rare. Although EAC incidence has been dramatically increased in Europe and North America at an alarming rate surpassing any other solid malignancies [3, 4], ESCC remains more prevalent worldwide, accounting for >90% of esophageal cancers and displaying high incidence in Central and East Asia (in particular, China), Sub-Saharan East to South Africa and a part of South America (Brazil) [5].

ESCC arises via malignant transformation of esophageal epithelial cells with activation of epidermal growth factor receptor (EGFR), PI3K and cyclin D1 oncogenes and mutations in *TP53* and $p16^{INK4A}$ tumor suppressor genes representing common genetic alterations [6–10]. By contrast, EAC develops as esophageal epithelium is displaced by specialized intestinal columnar mucosa. This metaplastic condition, termed Barrett's esophagus (BE), arises in the setting of gastroesophageal reflux disease (GERD) and predisposes individuals to EAC. Genome wide association studies have identified multiple disease susceptibility loci in both ESCC [11–15] and EAC [16, 17], suggesting complex interplays between genetic and environmental factors. Risk factors for ESCC include tobacco smoking and alcohol drinking while reflux esophagitis and obesity are the predominate risk factors for EAC [18–24]. Both ESCC and EAC are uncommon in young people although young onset ESCC has been linked to rare genetic conditions including Fanconi anemia [25–27], tylosis [28] and autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED) [29, 30]. APECED features esophageal candida infection.

In addition to these established risk factors, various other infectious agents have been linked to the pathogenesis of esophageal diseases, including cancer. Herein we describe the current understanding of the role of infectious agents in esophageal carcinogenesis. Characterization of the esophageal microbiome under homeostatic conditions as well as in the context of esophageal carcinogenesis will be reviewed. Additionally, studies evaluating the influence of the oral and gastric microflora as they relate to esophageal cancer will be discussed. Specific bacterial, viral, fungal and parasitic agents that have been linked to esophageal carcinogenesis will be delineated with particular focus on *Helicobacter pylori*, Human Papilloma Virus, *Herpesviridae*, Human Immunodeficiency Virus (HIV) and, which are among the most well-characterized in terms of influencing carcinogenesis in the esophagus.

3.2 Bacteria and Esophageal Carcinoma

3.2.1 Esophageal Microbiome

Numerous clinical studies have characterized the bacterial flora of the esophagus under normal conditions as well as in the context of esophageal pathology. Initial investigations into the esophageal microbiome relying upon culturing of aspirated esophageal secretions demonstrated limited bacterial diversity *Streptococci being* identified most frequently [31–33]. Comparison of culture growth from oral and esophageal aspirates further revealed similarity in microbial composition between these two sites [34], supporting the notion that the esophagus was likely to be passively colonized via transient passage of oral secretions. With the advent of high-throughput DNA sequencing technology, however, culture-independent characterization of the esophageal microbiome identified a diverse bacterial landscape that is distinct from that of the oral cavity. By sequencing of the 16S ribosomal RNA (rRNA) gene in endoscopic biopsy specimens from four individuals without esophageal disease, Pei and colleagues identifying 95 unique bacterial taxa belonging to six predominate phyla: Firmicutes (70%), Bacteroides (20%), Actinobacteria (4%), Proteobacteria (2%), Fusobacteria (2%) and TM7 (1%) [35]. Consistent with culture-based studies, the *Streptococcus* genus was most frequently represented, making up 39% of total clones, with enrichment of *Prevotella* (17%) and *Veillonella* (14%) also noted [35]. *Spirochetes*, commonly found in the oral cavity [36], was absent in the esophagus [35], indicating the esophageal microflora is distinct from that of the oral cavity. Subsequent investigations have validated these findings in normal patient cohorts using tissue specimens obtained through endoscopic biopsy and the Enterotest [37–40], a capsule-based string technology.

Alterations in the global esophageal microbiome as well as the level of specific bacterial species have been characterized in the context of esophageal carcinogenesis. A comparison of microbial composition in 142 dysplastic patients and 191 healthy controls from China, demonstrated that lower microbial diversity in the esophagus is associated with esophageal squamous dysplasia [41], the premalignant precursor to ESCC. Significant positive associations between the gram-negative bacterial species *Fusobacterium nucleatum* and tumor stage-specific survival have been demonstrated in ESCC patients [42]. Across 20 formalin-fixed paraffin-embedded ESCC tissue specimens, *F. nucleatum* positivity was detected in 20% of tumors and 5% of adjacent non-tumor mucosa with expression noted to be highest at superficial areas and lowest at invasive tumor fronts [42]. *F. nucleatum*-positive ESCC cancer tissues further displayed an enrichment signature that included "cytokine-cytokine receptor interaction" and significantly correlated with C-C motif chemokine ligand (CCL) 20 expression, suggesting that the pathogen may contribute to acquisition of aggressive tumor behavior via cytokine signaling [42]. The FadA virulence factor expressed on the surface of *F. nucleatum* promotes colorectal tumor growth by activating E-cadherin/Wnt signaling [43]. Although

Wnt signaling has been implicated in ESCC pathogenesis [44], whether this relates to *F. nucleatum* has yet to be explored. The gram-negative bacterial species *Streptococcus anginosus* has also been identified in esophageal dysplasia and carcinoma lesions [45–47]. While infection of the ESCC cell line TE6 with *S. anginosus* induced expression of the CXC-chemokine genes *IL18* and *CXCL1* [46], further investigation is required to examine the functional significance of *S. anginosus* in ESCC pathobiology.

Several studies have identified alterations in the esophageal microbiome in the context of EAC, as well as its precursor conditions GERD and BE. An initial characterization of Gram-staining via retrospective analysis of archived mucosal biopsy specimens revealed increased microbial colonization, predominately by Gram-positive cocci, in BE as compared to controls [48]. Clustering analysis of bacterial 16S rRNA sequencing data of distal esophageal biopsies from ten healthy controls, 12 GERD patients and ten BE patients further identified two microbiome subtypes [49]: the Type I microbiome was enriched for *Streptococci* and concentrated in healthy controls whereas the Type II microbiome exhibited enhanced diversity with a greater proportion of Gram-negative anaerobes, including *Veillonella, Neisseria*, *Prevotella*, *Campylobacter*, *Porphyromonas*, *Fusobacterium*, and *Actinomyces*, and correlated with GERD (OR 15.4, 95% CI 1.5–161.0) and BE (OR 16.5, 95% CI 1.5–183.1) [49]. Although Gall and colleagues also detected *Streptococci* in BE, they described significant heterogeneity in the abundance of this genus across BE patients and further reported that *Streptococcus*:*Prevotella* ratio is associated with waist-to-hip ratio and hiatal hernia length, two established BE risk factors [50]. Enhanced bacterial diversity in BE patients as compared to normal controls has been independently confirmed [51, 52]. By contrast, when compared to controls, EAC displayed decreased richness in microbial content [53], but increased relative abundance of *Bifidobacteria*, *Bacteroides*, *Fusobacteria*, *Veillonella*, *Staphylococcus* and *Lactobacilli* [54].

At the species level, *Streptococcus pneumoniae* was found to be more abundant in GERD and BE as compared to tumor-adjacent normal epithelium, dysplasia and EAC lesions [55], the latter of which EAC lesions featured enrichment for *Lactobacillus fermentum*. *Campylobacter concisus and Campylobacter rectus* were uniquely detected in 57% of BE patients [51]. Increased abundance of *C. concisus* was confirmed in BE patients as well as in those with GERD, and correlated with enhanced production of interleukin (IL)-18 [54], a positive effector of tumor cell proliferation, migration, metastasis and immune evasion in gastric cancer [56–58]. *C. concisus*-mediated induction of IL-18, as well as p53 and tumor necrosis factor (TNF)-α, was confirmed in BE cell lines *in vitro* [59]. *Escherichia coli* was also detected in BE and EAC, but absent in clinical specimens from tumor-adjacent normal epithelium, dysplasia and GERD [55]. Emerging evidence from human population studies and murine models support *E. coli* as a tumor promoting factor in colorectal cancer; however, what role, if any, *E. coli* plays in EAC has yet to be determined [60–63].

3.2.2 Oral Microbiome

Epidemiological studies have demonstrated a positive correlation between poor oral health and esophageal cancer risk [64–66], raising the possibility that alterations in the oral microbiota may influence esophageal carcinogenesis. Supporting such a premise, a retrospective case control study in a cohort from a high-risk area in China revealed decreased microbial diversity in the saliva from ESCC cases (n = 87) as compared to normal controls (n = 85) or patients with esophageal dysplasia (n = 63) [67]. Specifically, saliva from ESCC cases displayed decreased carriage of the bacterial genera *Lautropia*, *Bulleidia*, *Catonella*, *Corynebacterium*, *Moryella*, *Peptococcus* and *Cardiobacterium.* Despite an overall decrease in these bacterial genera, ESCC cases exhibited higher abundance of *Prevotella*, *Streptococcus* and *Porphyromonas* genera in their saliva as compared to non-ESCC controls [67], indicating that a shift in the oral microflora may accompany esophageal carcinogenesis. An additional case control study prospectively evaluated oral bacterial species in pre-diagnostic mouthwash samples from EAC (n = 81) or ESCC (n = 25) patients and matched controls (n = 160/50), finding that depletion of the commensal genus *Neisseria* and *Streptococcus pneumoniae* as well as bacterial carotenoid production were associated with decreased EAC risk [68]. Elevated risk of EAC and ESCC was demonstrated in relation to *Tannerella forsythia* and *Porphyromonas gingivalis,* respectively [68]. Notably, *T. forsythia* and *P. gingivalis* are among the oral pathogens most strongly associated with severe periodontitis. Further supporting a role for *P. gingivalis* in ESCC pathogenesis, immunohistochemistry (IHC) staining detected *P. gingivalis* in 61% of ESCC tumor specimens and 12% of tumor-adjacent tissues while failing to identify the pathogen in normal mucosa [69]. In ESCC lesions, *P. gingivalis* antigen expression negatively correlated with patient survival and lymph node metastasis [69].

3.2.3 Gastric Microbiome

A link between gastric biology and esophageal cancer is supported by observational studies demonstrating that gastric fundic atrophy serves as an independent risk factor for ESCC [70–78]. To explore the relationship between the gastric microbiome and ESCC, Nasrollahzadeh and colleagues compared the gastric fundal microbiome pattern in ESCC cases, consisting of ESCC stage I–II and esophageal squamous dysplasia, with that of either healthy controls or patients with mid-esophagus esophagitis [79]. Consistent with published findings [80], the most common phyla in gastric mucosa were *Proteobacteria*, *Firmicutes*, *Bacteroidetes*, *Actinobacteria* and *Fusobacteria* with phyla composition consistent across cases and controls. ESCC cases also exhibited increased abundance of gastric fundal bacteria of the *Clostridiales* and *Erysipelotrichales* orders, both belonging to the *Firmicutes* phylum, as compared to either healthy or esophagitis controls [79]. Principal coordinate

analysis of sequencing data identified distinct patterns of gastric microbiota between ESCC cases and healthy controls [79]. No such differences were found when comparing healthy controls with esophagitis controls, suggesting that alterations in the gastric microbiome may occur specifically in the context of ESCC.

3.2.4 Helicobacter pylori

The *Proteobacteria* species *H. pylori* is the primary causative factor in stomach cancer, attributable to nearly 90% of gastric cancers worldwide [81]. The incidence of gastric cancer has dramatically declined in the last 30 years as antibiotic use has become widespread in clinical practice. Conversely, esophageal cancer has become increasingly prevalent during this period with numerous epidemiological studies supporting an inverse correlation between *H. pylori* infection and incidence of both BE and EAC [82–95]. Notably, the relationship between *H. pylori* and GERD remains inconclusive [96–102]. *H. pylori*-mediated suppression of BE and EAC is largely attributed to pathogen-induced gastric atrophy and resultant suppression of gastric acid secretion [103] with additional potential contributory mechanisms including pathogen-mediated suppression of aneuploidy, induction of tumor cell apoptosis, and disruption of the local microbiome [50, 104, 105]. The apparent protective nature of *H. pylori* with regard to EAC is further highlighted in that several studies have reported occurrence of GERD and its sequelae following pathogen eradication. A prospective study evaluating 105 patients, found reflux esophagitis in 11 (0.5%) patients at 7 months following *H. pylori* eradication, noting a positive correlation between esophagitis and gastric acid secretion [106]. In patients receiving *H. pylori* treatment for duodenal ulcer therapy, incidence of reflux esophagitis within 3 years was 25.8% with successful *H. pylori* eradication and 13% with persistent infection [107]. Two independent case reports have further noted a newly developed EAC lesion and a case of BE thorough erosive esophagitis following *H. pylori* clearance [108, 109].

Despite the wealth of literature supporting a potential protective influence of *H. pylori* upon EAC, controversy remains with some studies failing to identify a significant relationship between the pathogen and EAC [110, 111]. There is potential that pathogen strain may contribute to conflicting findings as *H. pylori* strains that are positive for the virulence factor cytotoxin-associated gene (Cag) A are less likely to be associated with EAC [92, 95]. A role for *H. pylori* in promoting EAC was supported by experimental model systems. Exposure of the normal human esophageal cell line HET-1A to *H. pylori* extract augmented acid-induced molecular markers associated with intestinal metaplasia, including caudal homeobox protein 2, mucin 2 and cyclooxygenase 2 [112]. Moreover, functional studies in a rat model of chronic gastroesophageal reflux, demonstrated that esophageal *H. pylori* colonization enhanced inflammation as well as the incidence of BE and EAC

while colonization of the stomach with *H. pylori* failed to influence esophageal phenotypes [113, 114].

With regard to ESCC, numerous epidemiological studies have failed to delineate a significant association with *H. pylori* using multiple cohorts [86, 87, 93–95, 115]. In agreement with these studies, a South Africa-based descriptive case study series noted the prevalence of *H. pylori* in ESCC patients to be similar to that of general population [116]. Conversely, an inverse correlation between *H. pylori* and ESCC risk was identified by two independent studies via evaluation of pathogen seropositivity or prevalence in biopsy specimens [117, 118]. In two meta-analysis studies, association of ESCC with specific *H. pylori* strains revealed significant associations between CagA-positive *H. pylori* and ESCC upon risk stratification based upon study location with a protective effect noted in Eastern-based cohorts [95, 115]. Additionally, a marginally significant increase in ESCC risk was detected with CagA-positive *H. pylori* strains by Islami and colleagues via meta-analysis [86].

3.2.5 Targeting the Microbiome to Improve Esophageal Cancer Outcomes

In sum, the described studies indicate that the microbiota of the esophagus itself as well as that of surrounding organs may serve as effectors of esophageal carcinogenesis. While it is tempting to speculate that modulation of the microbiome may be an effective approach toward improving esophageal cancer prognosis and management, studies addressing causality are necessary to define functional roles for bacteria in esophageal carcinogenesis. While used commonly in clinical practice, broad spectrum antibiotics have the potential to dramatically skew the both local and organismal microflora with the potential for undesirable outcomes. In humans, a case control study featuring 6108 cases and 23,850 controls identified an increased risk of esophageal cancer in individuals reporting more than five courses of penicillin [119]. Additionally, the use of penicillin G and streptomycin in a rat esophagojejunostomy model of BE and EAC, failed to significantly influence tumor incidence [120]. Given that alterations in specific bacterial strains have been identified in the context of esophageal carcinogenesis, it is possible that targeted approaches to anti- or probiotic therapy may be effective while minimizing off-target effects. Additionally, modification of the microbial composition of the oral cavity and stomach may influence esophageal carcinogenesis. Indeed, an ongoing clinical trial (NCT02513784) aims to evaluate the influence of and oral chlorhexidine rinse upon the esophageal and gastric cardia microbiomes.

As our understanding of the complex relationship between bacteria and esophageal carcinogenesis grows, this knowledge may then help to inform future approaches toward manipulating the microbiome with the goal of improving esophageal cancer patient outcomes.

3.3 Viruses and Esophageal Carcinoma

3.3.1 Human Papilloma Viruses

Human papillomaviruses (HPVs) are a circular double-stranded DNA viruses that infect and replicate in cutaneous and mucosal epithelia [121]. Amongst the 120 known HPV genotypes, mucosal HPVs can be classified into 14 high-risk groups (e.g. types 16, 18, 31, 33, 45), six possibly high-risk groups, and 31 low-risk groups (e.g. types 6, 11), depending on their association with benign or malignant tumors in the cervix [122, 123]. The HPV genome is approximately 8 kB in size and comprises early and late regions that respectively encode six early proteins (E1, E2, E4, E5, E6 and E7) and two late (L1 and L2) proteins. HPV infection has been examined by serology for circulating anti-HPV antibodies directed against HPV-type specific E6 and E7 antigens [124–126] and viral particles [127]. In tissues and cells, HPVs have been detected via IHC for HPV-related antigens, *in situ* hybridization (ISH) or polymerase chain reaction (PCR) for HPV DNA. Amongst HPV-encoded gene products, E6 and E7 have been most extensively characterized with respect to their roles in malignant transformation of esophageal epithelial cells (keratinocytes). While both E6 and E7 may physically interact with multiple cellular proteins in HPV-infected cells, high risk HPV-derived E6 and E7 inactivate key tumor suppressor proteins TP53 [128] and RB [129] directly. Both E6 and E7 have been utilized to immortalize normal human esophageal keratinocytes [130] from which tumorigenic transformed ESCC cell line EN60 has been derived [131, 132].

In the cervix, HPV is found in approximately 90% and 75% of squamous cell carcinoma and adenocarcinoma, respectively. HPV16 is most prevalent (46–63%) followed by HPV18 (10–14%), 45 (2–8%), 31 (2–7%) and 33 (3–5%) in squamous cell carcinoma while HPV18 is predominant (37–41%) over 16 (26–36%) and 45 (5–7%) in adenocarcinoma [122]. In Denmark, a nationwide population-based cohort study comprising 83,008 women displaying cervical HPV colonization demonstrated increased risks for anal and ESCC (standardized incidence ratio 1.4, 95% CI 0.91–1.9 compared to the general population) during follow-up for a median of 14.9 years [133]. The prevalence of high-risk HPV in cervical cancer and head and neck cancer is nearly 90% [134] and 30% [135], respectively. Such a difference may be accounted for by the variable frequency in sexual transmission of HPV at the different anatomic sites.

HPV-induced non-neoplastic esophageal pathologies such as esophagitis has been rarely documented [136]. Esophageal squamous papilloma (ESP) is a relatively uncommon benign tumor with an estimated prevalence of 0.01–0.45% [137–145]. ESP is suspected as an early histologic lesion of ESCC as observed in chemical carcinogen-induced rodent ESCC models [146–148], including those carrying HPV16 E6 and E7 transgenic oncogenes targeted to oral and esophageal epithelia. HPV has been implicated in ESP and other esophageal benign lesions, such as hyperplasia where IHC detected HPV antigens in the nuclei of both superficial dyskeratotic cells and koilocytes, characteristic of HPV-infected squamous-cell

epithelia [149, 150]. ESP contains HPV DNA at a variable frequency (0–65%) with benign types of HPV being most commonly detected [137, 140, 144, 151–157]. High-risk HPV strains (HPV16 and HPV18) were detected in 57% of ESP patients in the United States (n = 21) [153]. Notably, the incidence of ESP associated with high-risk HPV infection has dramatically increased in the United States in the recent years. Indeed, a cross-sectional study of 60 ESP patients identified from 2000 to 2013 postulated a fourfold increase in the incidence of esophageal papilloma during this time period with 47% of patients displaying HPV16-positivity [158].

Studies identifying HPV DNA sequences in primary ESCC and squamous dysplasia, a premalignant lesion, first emerged in the 1990s. Esophageal biopsies from ESCC-adjacent mucosa isolated from patients from a high-incidence ESCC area in China revealed HPV DNA in both epithelial hyperplasia (36.1%, n = 51) and dysplasia (22.2%, n = 51) [159]. Amongst HPV types, HPV16 was most common (72.7%, n = 22) as was further validated upon evaluation of a larger patient cohort (n = 363) [160]. However, the prevalence of high-risk type HPV infection was significantly lower in ESCC patients than cervical cancer patients in Northern China where both ESCC and cervical SCC are highly endemic [161]. In this study, HPV16 accounted for >90% of HPV-positive lesions in both tumor types [161]. This was corroborated by a meta-analysis study demonstrating a relatively low HPV18 prevalence (<10%) in Chinese ESCC patients [162]. HPV DNA was detected by PCR in 42.9% of ESCC primary tumors and 66.7% of adjacent mucosa in South African patients (n = 14) where HPV was detected in 15% of esophageal mucosa from non-cancer control patients (n = 41) [163]. Esophageal HPV infection was detected in 41.7% French patients with ESCC (n = 12) where HPV16 and HPV18 were detected in a subset of tumors by dot blotting [164]. In Japanese patients, HPV16 and HPV18 were detected by ISH in 14.1% and 20.1% (n = 71), respectively, of surgically resected ESCC tumors [165]. While HPV types with less-defined pathological significance (e.g. HPV30) were detected in ~10% of ESCC tumors [166, 167], other studies reported low HPV prevalence in ESCC and its precursor lesions even within identical ethnic groups [168–170]. Indeed, HPV DNA detection rate by PCR varied from 0% to 90% in human subjects within highly endemic areas in China, calling for a more rigorous approach in sample handling [171]. In addition to sporadic ESCC, HPV has been explored in Fanconi anemia, a rare hematopoietic genetic disease in which early-onset ESCC and other SCCs arise in individuals who have survived bone marrow failure or leukemia as a result of bone marrow transplantation coupled with chemotherapy. In a United States study, high-risk HPV was detected in 84% of Fanconi anemia-related SCC tumors (n = 25) [172]; however, only 10% of similar tumors (n = 21) were positive for high-risk HPV in an European study [26] despite similar virus detection by PCR.

To date, >150 studies have addressed HPV prevalence in ESCC worldwide, permitting subsequent meta-analyses that confirmed the overall prevalence of HPV in ESCC to be 20–35% and provided evidence for HPV as an ESCC a risk factor [173–177]. A meta-analysis of 33 randomized studies focusing upon the relationship between ESCC and the high-risk HPV strains 16 and 18, determined overall HPV prevalence to be 46.5% and 26.2% in the cancer and control groups,

respectively [178]. Although substantial geographical variance exists with regard to the relationship between HPV prevalence and ESCC, epidemiological evidence supports an etiological role for the virus specifically in high-incidence areas, including Asia (particularly China being high-risk areas) and Africa [177, 179, 180]. HPV16, but not HPV18, infection appeared to be the foremost risk for ESCC [181]. Interestingly, the association between HPV and ESCC has been found to be stronger in countries with low to medium ESCC incidence compared to the regions of high ESCC incidence [176].

As substantially better therapy response, low progression risk, and favorable prognosis have been reported in patients with HPV-positive head and neck squamous cell carcinoma [182, 183], clinical outcome has been investigated in HPV-infected ESCC patients. Several studies have linked HPV infection to improved chemoradiation therapy response despite disease stage [184, 185] and identified HPV infection as an independent predictor of favorable prognosis in ESCC patients [186], especially following chemoradiation therapy [187]. A recent meta-analysis study, however, failed to detect a better prognosis associated with HPV infection [188] although such a conclusion may have been confounded by patients with low-risk HPV infection.

Genomic sequencing analyses revealed the presence of HPV DNA as an integrated form in ESCC specimens [189]. HPV16 DNA integration has been documented at multiple human chromosome sites [190] with integration rate in tumor tissues (93.4%) nearly twice as high as that of tumor-adjacent mucosa (50.9%) [191]. Greater than 90% of HPV-positive primary ESCC tumor samples (n = 30) carry integrated viral DNA and display augmented expression of E6 and E7 oncoproteins via disruption of the HPV E2 gene [192]. In agreement, HPV16 and HPV18 can replicate in ESCC cells independent of E1 and E2 proteins via host nuclear factors [193]. High risk-HPV infection and genomic integration have been linked to activation of telomerase and telomere maintenance in ESCC cells [194]. HPV18 DNA integration has been described in ES9706 and EC109 ESCC cell lines [195–197].

HPV infection, especially HPV18 and HPV16, is found in cervical adenocarcinoma where HPV-positive patients are younger than HPV-negative patients [198]. Prevalence of HPV infection in esophageal adenocarcinoma (EAC) and its histologic precursor lesions remain elusive. In a cross-sectional study with prospectively enrolled male patients undergoing upper endoscopy in the United States, HPV was not detected in non-dysplastic BE by IHC or PCR in the presence of adequate quality control [199]. In an Australian cohort, however, HPV DNA was detected in 31% of patients (n = 261) with non-dysplastic BE, dysplastic BE or EAC where HPV prevalence appeared to be more frequent in Barrett's dysplasia (68.8%) and EAC (66.7%) compared to BE (22.1%) or normal mucosa (18%) [200]. In a follow up study, the authors reported HPV prevalence in 25.7% of patients (n = 218) where HPV16 was most common (75%) followed by HPV18 (23.2%) [201]. In this study, HPV transcripts (RNA) were detected in dysplasia or EAC, but not BE. Moreover, HPV-positive samples were characterized by the absence of *TP53* mutations concurrent with low protein expression of TP53 and RB proteins, which were targeted by HPV E6 and E7 proteins, respectively [201].

DNA sequencing of EAC tumors revealed the absence of *TP53* mutations and 50% fewer non-silent somatic mutations in cancer driver genes in high-risk HPV-positive tumors (n = 4) compared to HPV-negative (n = 8) tumors [202]; however, low HPV prevalence (0–10%) in EAC was noted in other studies despite geographical locations including Australia [203] and China [204]. A recent meta-analysis encompassing 30 studies determined the pooled prevalence of HPV to be 26% and 13% in BE and primary EAC, respectively. Moreover, HPV prevalence was found to be higher in patients with EAC than healthy controls. Considerable between-study variation as well as concerns related to relatively small sample sizes and detection methods represent limitations of the current literature that must be addressed to define the true prevalence of HPV in patients with BE and EAC [205].

3.3.2 Herpesviridae

Herpesviridae is a large family of DNA viruses, including herpes simplex viruses (HSV)1 and HSV2, Epstein-Barr virus (EBV), Cytomegalovirus (CMV), varicella-zoster virus (VZV), and Kaposi's sarcoma-associated herpesvirus (aka HHV-8), that cause a variety of human diseases. A recent bioinformatics study mined Cancer Genome Atlas RNA-Sequencing data representing 6813 human tumors and 559 adjacent normal samples across 23 cancer types to identify 505 virus-positive tumor samples [206]. Amongst gastrointestinal cancers, *herpesviridae,* including EBV and CMV, appeared to be the most prevalent viruses with significantly higher abundances in tumor versus adjacent normal samples [206]. *Herpesviridae* undergo latent infection. With or without established oncogenic properties, several *Herpesviridae* family members have been considered in the pathogenesis of esophageal cancers owing to their affinity for stratified squamous epithelia, including oral, esophageal and anogenital mucosa, and saliva-mediated transmission.

3.3.2.1 Epstein-Barr Virus

Amongst *herpesviridae,* EBV has been most extensively explored in esophageal cancers to date. EBV may cause acquired immunodeficiency syndromes (AIDS)-associated esophageal ulceration [207] and esophageal lymphoproliferative disorder in immunosuppressed patients [208]. EBV has been detected in tumors by PCR for EBV genomic DNA [170, 209–221], in situ hybridization (ISH) for EBV-encoded small RNA (EBER) [211, 214, 216, 219–228] or IHC for EBV-encoded latent membrane protein-1 (LMP1) or EBV nuclear antigen (EBNA) [214, 221, 224, 227, 229]. In most studies, EBV DNA and other markers were rarely detected, if any, in primary tumors of ESCC from patients in Japan, Korea, China, Pakistan, Russia and Germany [170, 209, 210, 212, 214–216, 220, 222, 223, 225, 226, 229–231]. EBV was also undetectable in primary EAC tumors from patients in German (n = 162) [228], French (n = 40) [221] and Korea (n = 3) [226]. One study in

Taiwanese patients reported EBV DNA in 35.5% of primary ESCC tumors (n = 31) concurrent with EBER expression; although, LMP-1 expression was undetectable by IHC in this study [214]. Another study described the presence of EBV DNA in 30% of ESCC lesions (n = 70) in Chinese patients [213]. Two additional studies reported EBV DNA detection in 35% of ESCC (n = 23) and 36% of EAC (n = 14) in German cohorts [217] and 47% of EAC (n = 17) in British cohorts [218]. Meta-analysis was performed on five studies [218, 221, 226, 228, 231] revealing 6% (95% CI 0–27%) EBV prevalence in EAC [205]. Most of these studies failed to localize EBV within heterogeneous tumor tissues. A few studies have localized EBV exclusively in tumor-infiltrating lymphocytes, but not cancer cells themselves [211, 220, 225, 226]. One of these studies show a significant correlation between the presence of EBV and the degree of lymphocyte infiltration in ESCC tumor stroma [224]. In this study, the authors examined primary ESCC tumors from 164 Chinese patients to find that EBV EBER and LMP-1 were present in 6.7% and 6.1%, respectively, of poorly-differentiated or undifferentiated tumors, but not well-differentiated or moderately-differentiated tumors [224]. Thus, there are cases in which EBV has been documented within cancer cells [224, 227]. Additionally, EBV was detected in a rare form of esophageal cancer with a lymphoid stroma reminiscent of nasopharyngeal lymphoepithelioma in three reported cases of Japanese patients [219, 227, 230]. In summary, current evidence suggest that EBV may have a pathogenic role in a rare subset of esophageal cancers, but not in the majority of ESCC and EAC.

3.3.2.2 Herpes Simplex Virus

While both HSV1 and HSV2 are the causative agents of oral and genital mucosal herpes, HSV infection may also cause esophagitis, albeit uncommon. Although HSV-induced esophagitis is a self-limited disease, it may be reactivated occasionally following primary infection [232]. Additionally, HSV causes ulcerative esophagitis in immunocompromised individuals [233]. Esophageal tropism, transmission via saliva and oncogenic potential of HSV [234] have prompted investigation of the role of HSV in esophageal cancers; however, only a few studies are presently available regarding the relationship between HSV and esophageal cancers.

HSV1 and HSV2 have been detected in tissues by IHC for specific viral antigens and ISH for viral genomic DNA [224]. In a study of 164 ESCC tumor samples from patients in Shantou, Guangdong, one of the highest ESCC endemic areas in China, HSV DNA was detected in 31.7% of the tumors while HSV1 and HSV2 antigens were detected in 17.1% and 23.8% of the tumors, respectively [224]. HSV was more frequently detected in well-differentiated (41.9%, n = 43) or moderately-differentiated (35.9%, n = 78) tumors compared to poorly-differentiated (13.3%, n = 30) or undifferentiated (15.4%, n = 13) lesions [224]. An additional study based in Shantou reported 30% HSV1-positivity by PCR in 70 esophageal cancer samples [213]; however, a Chinese study on 103 patients from another ESCC endemic area in Northern China reported the absence of *herpesviridae* including HSV, EBV and CMV in ESCC lesions [229], indicating the possible geographical variation in the etiological role of these viruses in ESCC.

3.3.2.3 Cytomegalovirus

CMV also causes esophagitis which is diagnosed by upper endoscopy and a serologic test for anti-CMV Immunoglobulin (Ig)G and IgM; however, CMV has not been detected in primary ESCC [213, 229] or EAC [218] tumors where CMV was examined by ISH or PCR for viral genomic DNA or IHC for a viral antigen; however, a study mining the Cancer Genome Atlas RNA-sequencing data detected not only HSV1 and EBV but also CMV in 3–4% of esophageal cancer samples [206].

3.3.2.4 Varicella-Zoster Virus

VZV causes chickenpox upon primary infection. Following chickenpox recovery, VZV persists for years as a latent form in nerve ganglia until reactivation which culminates in neurological conditions. Although VZV has not been directly determined in esophageal cancers, VZV may cause esophageal achalasia, an esophageal motility disorder [235, 236]. Interestingly, achalasia has been considered as a risk factor for esophageal cancers [237]. Additionally, young onset intestinal metaplasia (BE) has been reported in an infant with congenital varicella syndrome as a rare complication of VZV infection during pregnancy [238]. It seems unlikely that VZV directly causes intestinal metaplasia; however, VZV infection may affect esophageal motility to allow gastroesophageal reflux, which in turn facilitates intestinal metaplasia.

3.3.3 Human Immunodeficiency Virus

Benign esophageal lesions are found in nearly 50% of immunocompromised individuals with AIDS [239]. They include mucosal candidiasis [240, 241], HSV-related herpetic esophagitis [242], CMV-related esophagitis [243] and idiopathic ulcerative esophagitis [244]. HIV infection may facilitate tumor development and progression by increasing opportunistic infection of oncogenic viruses such as HPV and EBV while suppressing anti-tumor immunity. Additionally, HIV-infected individuals are prone to other cancer risk factors such as tobacco smoking. AIDS-defining malignant neoplasms (i.e. non-Hodgkin lymphoma and Kaposi's sarcoma) have been reported in the esophagus [245–247], albeit uncommon. HIV-infected individuals show a high incidence of a broad spectrum of non-AIDS-defining cancers, including oropharyngeal and anogenital cancers, most of which are squamous cell carcinomas [248–252]; however, the incidence of esophageal cancers amongst the HIV-infected population is not elevated compared to the general population in the United States [253]. HIV infection has not been linked to EAC or its precursor lesions (BE) to date.

More than 60% of world population with HIV live in sub-Saharan Africa according to the statistics of the Joint United Nations Programme on HIV/AIDS (UNAIDS). ESCC is highly endemic in this area [254, 255]. A study on 195 South African ESCC patients demonstrated that 22.6% were HIV-positive. Interestingly, HIV-positive

patients were significantly younger than those without HIV infection, suggesting that HIV infection may accelerate ESCC development and/or progression [256]. In a Zambian case-control study of 122 ESCC and 70 individuals with normal esophageal mucosa, HIV infection appeared to be an independent risk factor for ESCC and this was further enhanced in adults under 60 years. In this study, tobacco smoking and domestic smoke exposure from cooking, but not HPV infection or alcohol consumption, were found to increase the odds of ESCC development [257].

ESCC and head and neck squamous cell carcinoma (HNSCC) arise often in a synchronous or metachronous fashion [258–262]. HNSCC is detected at younger age and advanced stages in individuals with HIV infection compared to those without [263]. HIV infection is highly associated with unique multinucleated giant tumor cells in HNSCC with or without concurrent infection of HPV, EBV, HSV1 or HSV2 [264]. The TP53 tumor suppressor protein interacts with HIV-encoded viral proteins [265–267] including Nef protein which has been shown to shorten TP53 protein half-life to suppress TP53-dependent transcription and apoptosis [267]. Interestingly, analyses of HNSCC in HIV-positive patients demonstrated a unique pattern of gene mutations compared to HNSCC in HIV-negative patients and that *TP53* mutation was significantly infrequent in HIV-positive HNSCC [268], suggesting a unique oncogenic role of HIV in squamous cell carcinoma. Concurrent HPV infection has been documented in HIV-related HNSCC tumors [264]. Interestingly, HIV trans-activating regulatory protein TAT not only enhances the expression of HPV E6 and E7 oncogenes but also stimulates proliferation of oral keratinocytes carrying the HPV-16 genome [269]. Given anatomically continuous mucosa and shared genetic lesions including *TP53* mutations, future investigation is warranted for the common pathogenic role of HIV in squamous cell carcinomas, including HNSCC and ESCC.

3.4 Fungi and Esophageal Carcinoma

Infectious conditions of the esophagus are rare in immunocompetent individuals [270]. Amongst fungus-related pathologies, *Candida* esophagitis is common in HIV carriers or patients receiving antibiotics, acid suppressants (e.g. proton pump inhibitors), immunosuppressive agents (e.g. corticosteroid) and chemotherapy [271–273]. ESCC cancer patients often present with *Candida* colonization [274] which may reflect esophageal obstruction by tumors [275]. Earlier studies linked *Candida* species to oral leukoplakia, a histologic precursor lesion of oral squamous cell carcinoma [276] and suggested oral colonization of *Candida albicans*, the most common member of human gut microbiota, as an independent risk factor for oral cancer [277]; however, it remains unknown whether *Candida* infection promotes esophageal carcinogenesis.

A potential link between *Candida* infection and oral and esophageal squamous cell carcinomas has been noted in a rare genetic disease condition known as chronic mucocutaneous candidiasis (CMC) [30, 278–280], where reported cases, albeit

sporadic, show young-onset squamous cell carcinomas without common risk factors such as tobacco smoking and alcohol drinking. CMC features persistent or recurring *Candida* infection of the skin, nails and oropharyngeal, esophageal and genital mucosae in affected individuals [281]. CMC is associated with multiple immunological disorders and related conditions such as IgA deficiency, autosomal dominant hyper-IgE syndrome, autoimmune polyendocrinopathy, hypothyroidism and hepatitis. Genetic causes linked to CMC include IL-17 receptor A (IL-17RA) deficiency [282], gain-of-function mutations in signal transducer and activator of transcription (STAT)-1 [283, 284], STAT3 deficiency [285], and retinoic acid-related orphan receptors γ deficiency [286]. In a Finish cohort of CMC associated with autoimmune polyendocrinopathy, six out of 92 patients (6.5%) were found to have young-onset oral or esophageal squamous cell carcinoma although four out of six of these cancer patients smoked for >15 years [29]. It is unclear whether *Candida* colonization itself, perturbed cytokine-mediated signaling pathways in CMC, or both have a direct role in malignant transformation of oral and esophageal epithelial cells.

The potential mechanisms that *Candida albicans* may promote carcinogenesis include production of acetaldehyde [287], a major human carcinogen. Moreover, live *Candida albicans* facilitates generation of the esophagus-specific carcinogen, benzylmethylnitrosamine (aka N-nitroso-N-methylbenzylamine) in culture [288].

3.5 Parasites and Esophageal Carcinoma

Presently, *Trypanosoma cruz*i represents the single parasitic agent with a potential link to esophageal carcinoma. This protozoan flagellate species is the etiological agent of Chagas disease, a tropical parasitic disease affecting the nervous system, heart and gastrointestinal tract. Among the digestive manifestations of Chagas disease are achalasia and subsequent megaesophagus [289], which are attributed to dysfunction of enteric motor neurons. Both idiopathic and Chagasic megaesophagus are associated with 0.4–10% enhanced risk of ESCC [290, 291], suggesting that megaesophagus is the likely effector of enhanced ESCC risk in the context of Chagas disease.

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Chapter 4
Viruses and Glioblastoma: Affliction or Opportunity?

Haidn Foster and Charles S. Cobbs

Abstract Herpesviruses, polyomaviruses, and papillomaviruses have all been detected in glioblastoma cells and/or cell lines. Our group first published evidence of human cytomegalovirus (CMV), a herpesvirus, in glioblastoma specimens from immunocompetent patients in 2002. However, the discovery of CMV and other viruses in glioblastoma has met with controversy following several studies that failed to detect viral particles in GBM. Here we summarize the known relationships between viruses and malignant gliomas, including viral detection in GBM, the oncomodulatory effects of GBM-associated viruses, and the novel ways by which investigators are targeting viruses for the treatment of glioblastoma.

Keywords Cytomegalovirus · Glioblastoma · Herpesvirus · Polyomavirus · Papillomavirus

Abbreviations (Laboratory assay abbreviations listed in Table 4.2)

BKV	B.K. virus
CNS	Central nervous system
CMV	Cytomegalovirus
DC	Dendritic cell
EBV	Epstein-Barr virus
GBM	Glioblastoma

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HHV	Human herpesvirus
HPV	Human papillomavirus
IE	Immediate-early
JCV	John Cunningham virus
Tag	Large tumor antigen
MGMT	O^6-Methylguanine-DNA-methyltransferase
pp	Phosphoprotein
PDGFRα	Platelet-derived growth factor receptor alpha
rGBM	Recurrent GBM
SV40	Simian virus 40
tag	Small tumor antigen
TMZ	Temozolomide

4.1 Introduction to Glioblastoma

Glioblastoma, a WHO grade IV astrocytoma, is the most common and aggressive cancer of the central nervous system, comprising approximately 47% of all malignant CNS tumors [1]. The age-adjusted prevalence of GBM in the United States is 6.46 per 100,000 population, though the disease is more common among whites, men, and the elderly. The current standard of care for glioblastoma treatment consists of maximal surgical resection followed by radiotherapy and concomitant chemotherapy with the alkylating agent temozolomide. Recurrent GBMs are often additionally treated with bevacizumab, which inhibits the formation of tumor vasculature.

Glioblastoma can arise *de novo* (primary GBM) or develop from a lower-grade neoplasm (secondary GBM). Few etiological factors are known for GBM aside from ionizing radiation and rare genetic disorders such as Li-Fraumeni and Turcot type 1 syndromes [2]. Similarly few predictors of improved prognosis exist for GBM, aside from O^6-methylguanine-DNA-methyltransferase (MGMT) promoter methylation which silences the expression of AGT, a DNA repair protein that counteracts temozolomide's therapeutic DNA alkylation by removing alkyl adducts from certain bases [3]. Despite advances in treatment options, GBM remains incurable; median survival time with radiation and temozolomide is just 14.7 months; and only around 5% of patients are alive after five years [4, 5].

4.2 Detection of Viral Particles in Glioblastoma Cells

Several viruses—known as oncoviruses—are known to cause cancer (Table 4.1); however, to-date no virus has a proven causative role in the development of glioblastoma [2]. Even so, several classes of virus have been detected in resected glioblastoma tissue and GBM cell lines (Table 4.2), and infection by these viruses

Table 4.1 Oncoviruses and their associated cancer burdens [2, 6]

Virus	Classification	Global cancer burden	Associated malignancies
Human papillomavirus	Papillomavirus	5.2%	• Cervical cancer • Oropharyngeal cancer • Anogenital cancer (vulva, vagina, penis, anus)
Hepatitis B and C viruses	Hepadnavirus	4.9%	• Hepatocellular carcinoma • Non-Hodgkin lymphoma[a]
Epstein-Barr virus	Herpesvirus	1.0%	• Hodgkin lymphoma • Non-Hodgkin lymphoma • Burkitt's lymphoma • Nasopharyngeal cancer
Kaposi sarcoma-associated herpesvirus	Herpesvirus	0.9%	• Kaposi sarcoma
Human T-cell lymphotropic virus type 1	Retrovirus	0.03%	• Non-Hodgkin lymphoma

[a]Caused by hepatitis C virus

has resulted in cell transformation, angiogenesis, and induction of stemness *in vitro* and in experimental animal models. Considered together with the observed correlations between viral infection levels and markers of GBM progression, many researchers have thus theorized an oncomodulatory or even oncogenic role for these viruses.

4.2.1 Herpesviruses

4.2.1.1 Epstein-Barr Virus

Globally, around 200,000 cancer cases per year—including Hodgkin lymphoma, Burkitt lymphoma, and nasopharyngeal and stomach cancers—are attributed to infection with Epstein-Barr virus [25]. Though few studies have interrogated the presence of EBV in glioblastoma, the virus was found in one experiment to be present in 24% of high-grade glioma cases by next-generation sequencing; however, its presence could not be confirmed by *in situ* hybridization [7].

4.2.1.2 Cytomegalovirus

A majority of adults in the United States are infected with human cytomegalovirus, a β-herpesvirus [26]. While infection is typically subclinical in immunocompetent persons, devastating disease can result from infection of immune-naïve or immunocompromised hosts such as infants, AIDS patients, and transplant recipients [27]. CMV is tropic to monocytes, macrophages, and dendritic cells, but has also been

Table 4.2 Studies evaluating the presence of viruses in glioblastoma

	Study	Positive	Tested	Percentage	Detection methods
Epstein-Barr virus	Cimino et al. [7]	5	21	24	NGS
Cytomegalovirus	Cobbs et al. [8]	22	22	100	IHC for IE1-72, pp65, and p52/76kD IE/EA; ISH for IE and total CMV genome; nPCR for UL55
	Lucas et al. [9]	25	49	51	IHC for pp65 and IE1 (8/49 GBM positive)
	Rahbar et al. [10]	79	80	99	IHC for IEA and LA (76/80 GBM positive); ISH for total CMV genome
	Scheurer et al. [11]	21	21	100	IHC for IE1-72
Human herpesvirus 6	Chi et al. [12]	7	14	50	nPCR
	Crawford et al. [40]	41	88	47	ISH for U57 major capsid protein
	Cuomo et al. [13]	14	31	45	nPCR for 287 bp outer fragment and 163 bp inner fragment of HHV-6 DNA; Southern blot hybridization for HHV-6B HindIII restriction site
	Luppi et al. [14]	5	13	38	PCR for 8.7 kb Hind III fragment
Simian virus 40	Caldarelli-Stefano et al. [15]	0	5	0	nPCR for Tag-coding region
	Huang et al. [16][a]	7	28	25	PCR for SV40 Tag-coding region; Southern blot hybridization
	Huang et al. [16][b]	13	22	59	PCR for SV40 Tag-coding region; Southern blot hybridization
	Kouhata et al. [17]	3	32	9	PCR for SV40 regulatory region; ISH for mRNA coding SV40 Tag
	Martini et al. [18]	10	30	33	PCR for amino-terminal Tag-coding sequence conserved in early region of SV40, JCV, and BKV; Southern blot hybridization with SV40 internal oligoprobe; RT-PCR; indirect IF with anti-SV40 Tag-specific Pab101 Mab
	Rollison et al. [19][c]	0	102	0	PCR for SV40 776; Southern blot hybridization with 32P-labeled whole genome plasmids
	Rollison et al. [19][d]	0	86	0	Real time qPCR for SV40

John Cunningham virus	Boldorini et al. [20]	7	13	54	nPCR for Tag-coding region; Southern-blot hybridization; PCR for viral protein (VP1)-coding region in Tag-coding-positive samples; nPCR for transcription control region (TCR) in Tag-coding-positive samples
	Caldarelli-Stefano et al. [15]	0	5	0	nPCR for Tag-coding region; IHC for JCV Tag
	Del Valle et al. [21]	12	21	57	PCR for Tag-coding region (amino terminal); Southern blot hybridization with radiolabeled JCV-specific oligonucleotide; IHC for Tag
	Huang et al. [16][a]	0	28	0	PCR for JCV Tag-coding region; Southern blot hybridization
	Huang et al. [16][b]	0	22	0	PCR for JCV Tag-coding region; Southern blot hybridization
	Muñoz-Mármol et al. [22]	1	18	6	PCR for Tag-coding region; Southern-blot hybridization; PCR for viral protein (VP3)-coding region; IHC for Tag and amino-terminal region common to all JCV early proteins
	Rollison et al. [19][c]	2	102	2	PCR for JCV Mad-1; Southern blot hybridization with 32P-labeled whole genome plasmids
	Rollison et al. [19][d]	0	86	0	Real time qPCR for JCV

(continued)

Table 4.2 (continued)

	Study	Positive	Tested	Percentage	Detection methods
B.K. virus	Caldarelli-Stefano et al. [15]	0	5	0	nPCR for Tag-coding region
	Corallini et al. [23]	9	18	50	Southern blot hybridization; dot blot hybridization for BKV RNA; indirect IF for Tag and Tag antibodies; hemagglutination and hemagglutination-inhibition with antibodies to BKV capsid antigens; ELISA for BKV Tag and BKV Tag antibodies
	Huang et al. [16][a]	1	28	4	PCR for BKV Tag-coding region; Southern blot hybridization
	Huang et al. [16][b]	0	22	0	PCR for BKV Tag-coding region; Southern blot hybridization
	Martini et al. [18]	28	30	93	PCR for amino-terminal Tag-coding sequence conserved in early region of SV40, JCV, and BKV; Southern blot hybridization with SV40 internal oligoprobe; RT-PCR; indirect IF with anti-SV40 Tag-specific Pab101 Mab
	Negrini et al. [24]	1	10	10	Southern blot hybridization with 32P-labeled BKV DNA probe
	Rollison et al. [19][c]	3	102	3	PCR for BKV Dunn; Southern blot hybridization with 32P-labeled whole genome plasmids
	Rollison et al. [19][d]	0	86	0	Real time qPCR for BKV Dunn
Human papillomavirus	Vidone et al. [56]	12	52	23	nPCR with MY/GP primers; CISH; IHC for capsidic protein L1

CISH chromogenic in situ hybridization, *ELISA* enzyme-linked immunosorbent assay, *IF* immunofluorescence, *IHC* immunohistochemistry, *ISH* in situ hybridization, *PCR* polymerase-chain reaction, *NGS* next generation sequencing, *nPCR* nested polymerase-chain reaction, *qPCR* quantitative polymerase-chain reaction, *RT* reverse transcription

[a]Primary GBM samples

[b]Secondary GBM samples

[c]Testing conducted at NINDS laboratory

[d]Testing conducted at Johns Hopkins laboratory

detected in malignancies of the brain, prostate, colon/rectum, and skin [28–31]. First reported in the GBM tumor cells of immunocompetent patients in 2002, CMV's association with glioblastoma has been the subject of considerable controversy in the years following [8]. Though many studies have confirmed the presence of CMV particles in a majority of GBM cells [9–11, 32, 33], recent experiments have been unable to detect any sign of CMV in GBM [34–36].

4.2.1.3 Human Herpesvirus 6

The roseolovirus HHV-6 is tropic to lymphocytes and neural cells including embryonic glia, and has been detected in glioblastoma and neuroblastoma cell lines [37–39]. HHV-6 has been found in 38–50% of GBM tumors and 0–67% of normal brain samples, and some investigators have proposed that HHV-6 may be no more prevalent in tumor than healthy brain [12–14, 40]. While such results could indicate incident laboratory contamination, a number of factors suggest against this possibility, including: the HHV-6A variant has been found at a higher frequency than HHV-6B in neoplastic tissues; peripheral blood lymphocytes from a given patient contained the same virus variant as the related tumor; and patients positive for HHV-6 DNA consistently had HHV-6-specific circulating antibodies [13].

4.2.2 Polyomaviruses

4.2.2.1 Simian Virus 40

Simian virus 40 induces intracranial tumors, among other neoplasms, in experimental animals and transforms murine and human cells *in vitro* [41–43]. Though SV40 is a monkey virus, it was iatrogenically introduced into the human population from 1955 to 1963 when contaminated polio vaccines were administered to the public. A correlation has since been established by some groups between higher incidence of intracranial tumors and vaccination with SV40-contaminated vaccine, while other groups have determined no such correlation exists [16, 44, 45]. A survey of 13 laboratory investigations revealed that primary brain tumor specimens were nearly four times more likely than controls to be infected with SV40 [46], and 9–59% of GBM tumors have tested positive for the virus [16–18]. In one instance, SV40-GBM viral particles with similarities to SV40-PML virus were also isolated from human GBM [47]. Still, some groups have been unable to find evidence of SV40 in GBM tissue [15, 19], and despite the virus' frequent detection in GBM cells and tumorigenic activity in animal models, one study determined that the degree of glioma malignancy was not correlated with presence of SV40 genome [48].

4.2.2.2 John Cunningham Virus

Over 75% of healthy adults have circulating antibodies to JCV and B.K. virus [49]. JCV is the etiological agent responsible for progressive multifocal leukoencephalopathy, a fatal demyelination disease caused by lytic infection of oligodendrocytes [50]. In addition to oligodendrocytes, JCV has been found in neurons and glial cells [51, 52], and owes its tropism to glial cells to transcription factors local to the cells which interact with the virus' early promoter elements to express JCV large tumor antigen, enabling completion of the viral lifecycle (reviewed in [53]). Though several studies have found JCV in only 0–6% of GBM tumors [15, 16, 19, 22], nonproductive infection has been detected in GBM from one patient via PCR and IHC and verified as a mutated version of the Mad-1 strain of JCV by sequencing the resulting amplified DNA [54], and a pair of experiments reported JCV in 54–57% of examined GBMs [20, 21].

4.2.2.3 B.K. Virus

B.K. virus is highly oncogenic in animals, and the virus is known to transform both monkey and human cells [23]. Low copy numbers of BKV have also been detected in human tumors of the brain and pancreatic islets, with ependymomas and choroid plexus papillomas ranking among the human tumors with the highest infection rates. Though BKV is rarely detected in GBM, with many reports of only 0–10% of tumor samples infected [15, 16, 19, 24], a pair of studies reported BKV in 50–93% of GBM tumors [18, 23].

4.2.3 Human Papillomavirus

Comprised of over 150 variants, the human papillomaviruses—in particular HPV16 and HPV18—are collectively associated with nearly 100% of the world's cervical carcinomas [55]. HPV additionally has a causative role in some oropharyngeal and anogenital cancers in both sexes. Though little information exists regarding HPV's presence in GBM, one study found the virus in 12 of 52 GBM tumor specimens [56].

4.3 Effects of Viral Infection on Glioblastoma Progression

There are several means by which viruses associated with GBM have been shown or theorized to have an oncomodulatory effect, including the direct transforming or malignancy-promoting action of viral antigens and polynucleotides (Fig. 4.1) as well as immunomodulatory effects such as inflammation and immunosuppression that result in a microenvironment conducive to tumor growth.

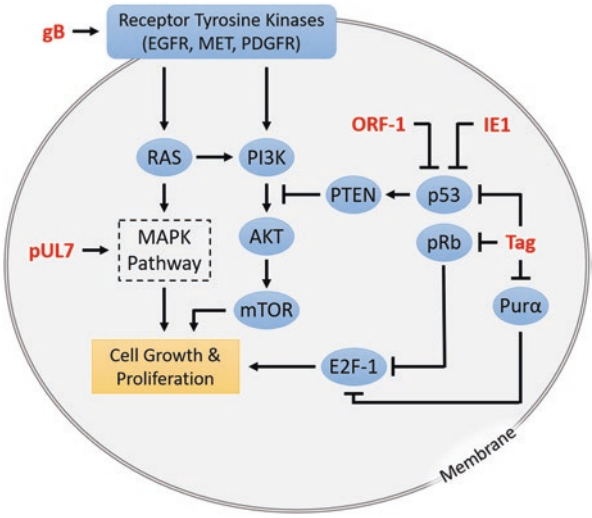

Fig. 4.1 Impact on cell signaling pathways by viral particles (labeled in red)

4.3.1 Oncomodulatory Proteins and Polynucleotides

4.3.1.1 Polyomavirus Large Tumor Antigen

Polyomaviruses SV40, JCV, and BKV are strongly tropic for glial cells *in vivo*, and have induced multiple brain neoplasms—including GBM and other astrocytomas, ependymomas, medulloblastomas, and choroid plexus tumors—in multiple experimental animal models such as mice, Syrian hamsters, and squirrel monkeys [57, 58]. The viruses' strongly oncogenic effect is caused primarily via expression of the viral large tumor antigen, a multifunctional protein that plays an integral role in polyomavirus replication and complexes with and at least partially inactivates the tumor suppressors p53, pRb (including pRb-family proteins p105, p107, and p130), and Purα—the cellular transcription regulator that induces JCV early gene transcription in glial cells by inactivating SRSF1, an alternative splicing factor that inhibits JCV activity [59–65]. When overexpressed, Purα works in a manner similar to pRb by binding the transcription factor E2F-1, inhibiting tumor cell growth [59].

BKV and SV40 Tag can both additionally induce chromosomal aberrations in human cells, a problem compounded by Tag's interference with p53's normal response to DNA damage [43, 66]. In the case of BKV, it was determined that Tag-mediated damage to the host cells' chromosomes occurred before immortalization, suggesting that such transformation is likely to have resulted from the chromosomal abnormalities rather than cause them, further elucidating the mechanism by which Tag transforms host cells [67].

Khalili et al.'s [68] "hit and run" theory describes how JCV Tag, along with other possible cofactors, may be necessary for tumor initiation but not progression, which could help to explain how mature malignancies with low viral copy numbers at the

time of detection may not reflect the full impact of initial infection on tumorigenesis. Still, the typically low expression of BKV Tag coupled with the fact that p53 still exhibits partial transcription when complexed with the molecule suggests that additional transforming events beyond polyomavirus infection may be required to give rise to human neoplasms [62].

4.3.1.2 HHV-6 ORF-1

Roseolovirus HHV-6 open reading frame 1 DNA has been detected in multiple human tumor specimens, including approximately 15% of glioblastomas [69]. ORF-1 is a transactivating gene that, by way of its protein inactivating p53-mediated transcription, transforms mouse fibroblasts and induces fibrosarcomas in nude mice. As p53 is one of the major tumor suppressors in healthy cells, ORF-1-mediated p53 inactivation is a likely mechanism by which HHV-6 could promote GBM tumor growth.

4.3.1.3 Cytomegalovirus Antigens and MicroRNA

Numerous investigations have determined that cytomegalovirus infection contributes to glioma stemness, invasiveness, and angiogenesis. Glioblastoma cell line U251, for example, exhibits increased cellular proliferation and higher levels of stemness markers Notch1 and Notch intracellular domain (NICD) upon infection with CMV [70]. Owing to its history of detection in myriad malignancies, CMV has some of the most extensively characterized oncomodulatory particles of any virus, summarized below.

The CMV immediate-early 1 protein is linked to glioblastoma stemness and proliferation. Stable expression of IE1 increases proliferation in the U87 and U118 glioblastoma cell lines and decreases p53mRNA and protein expression [71, 72]. Human glioma stem cells infected with a standard CMV strain also grew more readily as tumorspheres and xenografts than did those infected with a strain that does not express IE1 [73]. Murine gliomas artificially expressing IE1 additionally had higher levels of the stemness markers Sox2 and Nestin, mirroring the results seen in primary GBM cells in which CMV infection upregulates stemness indicators CD133, Notch1, Oct4, and Nestin [74]. Finally, 76% of primary GBM tumors were found to contain cells which coexpressed IE1 and CD133, and the degree of this coexpression was predictive of worse patient outcomes.

Detected in higher levels in $CD133^{+}$ cells, pp71 is another CMV protein that promotes stemness, angiogenesis, and inflammation via NFKB activation, resulting in the upregulation of stem cell factor [75]. In addition, CMV protein pUL7 induces IL-6, an inflammatory and proangiogenic cytokine, and activates the STAT3 and MAPK cancer signaling pathways [76], while the CMV-encoded chemokine receptor US28 increases glioma invasiveness and growth through induction of VEGF and activation of STAT3 [77]. Our group previously demonstrated that the CMV70-3P miRNA also promotes glioma stem cell stemness and proliferation [78].

Though most research has focused on the oncomodulatory effects of CMV particles within GBM cells post-infection, extracellular virions can also impact GBM progression via envelope glycoprotein B binding platelet-derived growth factor receptor alpha. This binding event increases glioma invasion *in vitro*, exacerbates tumor growth *in vivo*, and upregulates phospho-Akt levels, potentially allowing mutated cells to survive and proliferate [79]. Persistent stimulation of PDGFRα can also transform healthy neural stem cells, suggesting a way by which extracellular CMV could contribute to gliomagenesis [80].

4.3.2 Viral Cofactors

There is evidence that multiple viruses can interact to impact cancer growth and malignancy. Reviewed by Moens et al., the viral cofactor hypothesis posits that some non-oncogenic viruses found at increased levels in neoplastic tissue may promote the activity of true oncoviruses [81]. Early studies demonstrated that viral coinfection can enhance the activity of one or more of the viruses in question: adenovirus, for instance, activates the CMV immediate-early promoter, enabling viral transcription in otherwise quiescently infected cells [82].

CMV coinfection with JCV may also amplify the polyomavirus' oncomodulatory effects in GBM. Though CNS and tumor cells are permissive to HHV-6 infection, they support viral growth at reduced capacity and efficiency compared to lymphocytes, and there is evidence that HHV-6B establishes abortive infection in GBM cells *in vitro* [37, 83]. When coinfected with CMV, however, the virus' immediate-early transactivator 2 enables productive JCV replication in GBM cells and expression of JCV Tag, an antigen known for its oncomodulatory and transforming potential [84].

4.3.3 Viral-Mediated Immunomodulation

4.3.3.1 Inflammation

Chronic inflammation is associated with oncogenesis and increased malignancy in early neoplasias [85, 86]. This sort of long term, subacute (known as "smoldering") inflammation can be induced by viral infection and may result, for example, from polarization of macrophages to an inflammatory M1 phenotype via the release of TNF-α, IL-1, and IL-6 cytokines by monocytes infected with CMV [87]. HHV-6 also contributes to an inflammatory microenvironment by inducing the release of proinflammatory cytokines in the cyst fluids of glioma patients [12]. This has been verified *in vitro*, as infected astrocytes isolated from these patients release inflammatory IL-6 and IL-8 as well as immunosuppressive TGF-β and IL-10. Upon infection with HHV-6B and during periods of viral reactivation, the glioblastoma cell line U251 also releases inflammatory cytokines IL-6 and IL-1β [83].

4.3.3.2 Immunosuppression and Immune Evasion

In order to survive and replicate, many viruses have developed advanced means of subverting the body's immune response. Because the immune system is one of the ways by which the body naturally fights cancers, viral-mediated immunosuppression can result in more favorable conditions for tumor growth. cmvIL-10, a viral homolog of human IL-10, impedes immune response by hindering dendritic cell maturation and promoting the expression of programmed death ligand 1, resulting in CD8+ cytotoxic T-cell death and inhibition of apoptosis in immunosuppressive regulatory T-cells [88–92]. Immunosuppression is not the only means by which CMV assists infected cells in subverting the immune system: the virus also helps infected cells evade immune surveillance through the expression of UL18, a CMV homolog of MHC class I [93], and inhibition of MHC class II via production of US2, US3, and pp65 [94–96]. HHV-6 can similarly inhibit the host's immune response by promoting apoptosis in infected CD4+ T cells [97] and inducing T-regs [98]. Aside from the interaction of CMV gB with PDGFRα, the immunosuppressive effect of systemic viral infection is the most likely means by which viruses may impact GBM malignancy without infecting tumor cells, and this underexplored area warrants additional research.

4.4 Antivirals: A Suitable Therapy for GBM?

Antibody blockade and immunotherapy targeting known cancer-associated antigens such as EGFR/EGFRvIII and VEGF have had limited success in treating GBM. Antiviral approaches, however—including viral replication inhibition and viral antigen-targeting immunotherapy—have emerged as promising candidates for the treatment of glioblastoma.

4.4.1 Antiviral Agents

FDA-approved antiviral agents such as cidofovir and valganciclovir, a ganciclovir prodrug, have recently been employed to treat GBM. In the case of cidofovir, the drug caused apoptosis of GBM cells *in vitro* and exhibited antitumor effects in a murine xenograft model; however, cidofovir likely damages the DNA of rapidly-dividing tumor cells by inducing nonspecific double-stranded breaks as opposed to actually targeting viral replication [99]. On the other hand, valganciclovir inhibits cytomegalovirus replication, and in at least one study was found effective in treating GBM, pointing to localization of the virus in GBM as well as to the oncomodulatory effects exerted by viral infection and alleviated by the drug [100]. While the outcomes of this study were later questioned due to its incorporation of an additional cohort predisposed to better outcomes [101], the authors performed further statistical analysis showing benefit even after accounting for the added group [102].

4.4.2 Virus-Targeted Immunotherapy

Immunotherapy has emerged as a popular and effective method of treating myriad cancers, and investigators have since attempted to treat GBM specifically by targeting both cellular and viral antigens. *In vitro* studies have demonstrated the suitability of viral antigens as a target for GBM immunotherapy, as T cells extracted from CMV-infected glioblastoma patients and expanded in the presence of antigen-presenting cells—either transduced with IE1- and pp65-encoding adenovirus or pulsed with complete tumor RNA—lysed autologous CMV-positive GBM cells [103, 104].

Small clinical studies building on this laboratory work have had striking preliminary results. One trial (NCT00639639) using a combination of autologous DCs electroporated with pp65 mRNA and concomitant dose-intensified temozolomide significantly increased progression-free survival in GBM patients from 8.0 to 25.3 months and overall survival from 19.2 to 41.1 months (both $p < 0.0001$) [105]. The same group previously demonstrated that injection site prep with tetanus/diphtheria (Td) toxoid improves migration of DCs to vaccine site-draining lymph nodes and increases survival benefit—an approach that may prove useful to future investigators [106]. Adoptive cell therapy using CMV-targeting T cells has also demonstrated increased survival benefit among recurrent GBM patients. In one report, a patient exhibited reduced enhancing signal via MRI and 17+ months of clinical stability after four infusions of autologous T cells stimulated *ex vivo* with a combination of CMV epitopes and IL-2 [107]. Expanding on this initial success, the same investigators conducted a phase I clinical trial (ACTRN12609000338268) in which patients treated with autologous T cells trained against CMV achieved a median post-recurrence survival of 403 days [108].

4.5 Conclusion

The detection of viral particles in glioblastoma tumor cells and cell lines has a long and controversial history. While several classes of virus have been found in GBM, the variability of detection—including multiple studies failing to detect any significant viral presence in GBM—indicates the need for additional refinement and standardization of the methods used to detect low-level viral infection. Despite conflicting evidence regarding viral infection of GBM tumors, a host of oncomodulatory effects caused by viral particles in artificially-infected GBM cells have been characterized and several antiviral treatments have been confirmed to significantly increase progression-free and overall survival via seemingly specific, virus-targeting actions. Therefore, while infection with viruses such as CMV, HHV-6, and the polyomaviruses JCV, BKV, and SV40 is likely to have a deleterious effect on glioblastoma progression, these same viruses also make for an unprecedented target for treatment of a disease that until now has had limited therapeutic options.

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Chapter 5
The Microbiome and Its Contribution to Skin Cancer

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Abstract The skin is the largest human organ and its primary function is to provide a barrier against the external environment. Our world is inhabited by abundant and diverse microbial communities. Therefore, the skin necessarily comes in contact with myriad bacteria, viruses, and fungi. These interactions exert varying effects on symbiotic homeostasis and skin health. For some skin cancers, there is clear evidence of microbial etiological factors. In the skin, the viral pathogens Kaposi sarcoma herpesvirus/human herpesvirus 8 (KSHV/HHV8) and Merkel cell polyomavirus (McPyV) have a causal association with the skin cancers Kaposi sarcoma and Merkel cell carcinoma. Human papillomavirus (HPV) has an epidemiologic association with cutaneous squamous cell carcinoma (cSCC) but a mechanistic basis of viral carcinogenesis has been elusive. Recent advances in high-throughput sequencing technology have enabled investigators to attain increasingly comprehensive censuses of the skin metagenome through space and time. These "culture-free" molecular techniques have been employed to address fundamental questions pertaining to microbial etiologies of carcinogenesis. More recently, researchers are investigating the interplay between the immune system and skin bacterial and fungal microbiome diversity. These new findings may lead to future understandings of the skin microbial milieu and skin cancer risk.

Keywords Squamous cell carcinoma · Kaposi sarcoma · Merkel cell carcinoma

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5.1 Introduction

The first evidence that a microbe could cause cancer emerged in the early twentieth century. During this period, several investigators demonstrated that injecting tumor-derived cell-free filtrate was sufficient to induce carcinogenesis in the chicken *Gallus gallus*. Although these discoveries led to our current understanding of proto-oncogenes, they were initially dismissed as irrelevant to human cancer research because none of the known human cancers were contagious. It was not until decades later that Epstein, Achong and Barr discovered the first human oncovirus. They identified viral particles in cultured cells from Burkitt's lymphoma, and we now refer to this dsDNA herpesvirus as Epstein-Barr virus (EBV). Subsequent studies revealed that EBV infection is widespread amongst the human population, yet most people do not develop Burkitt's lymphoma. This suggests that cancer development is multifactorial and the carcinogenic potential of the pathogen manifests in a context-dependent manner. This is a departure from the classical view that a causal relationship between a specific pathogen and a disease may only be established if the Henle-Koch postulates are satisfied. One stipulation is the pathogen must be isolated, propagated in pure culture, and the disease must manifest when a lab animal is inoculated with the cultured pathogen. Another stipulation is that the pathogen cannot appear in healthy individuals as a fortuitous parasite disease. In the case of EBV, neither stipulation is feasible. Therefore, the requirements for establishing microbial disease causation were necessarily revised to incorporate new knowledge [1]. These revisions accommodated subsequent discovery of numerous oncoviruses, including those associated with skin cancer, namely: Kaposi sarcoma herpesvirus (KSHV) and Merkel cell polyomavirus (MCPyV). The necessary revisions to the Henle-Koch postulates are emblematic of our continually evolving understanding of the role of microbes in cancer development. Technological innovation in the field of high-throughput DNA sequencing has revolutionized our view of microbiota associated with the human body. Investigators have sampled the resident microbiota from multiple organs, including the skin [2]. We now know that the skin microbiome extends below the epidermis, is staggeringly complex, varies widely across space and time, yet is exquisitely unique to the individual, such that microbiome fingerprinting can be used forensically to identify individuals based on the microbes they carry [3]. The link between the skin microbiome and the immune system [4] may play an additional role in the association between immunosuppression and skin cancer risk.

5.2 Kaposi Sarcoma and Kaposi Sarcoma Herpesvirus

5.2.1 Discovery

Moritz Kaposi first described Kaposi sarcoma (KS) in 1872, over a century before a human herpesvirus was sequenced from KS lesions, establishing its infectious origin. Prior to this discovery, the pathophysiology of KS was largely speculative.

In the early nineteenth century, most cases of KS reported in the literature afflicted elderly Mediterranean men and followed a benign course. With the onset of the HIV epidemic in the 1980s, the narrative of KS changed as the number of cases reported in African men, women, and children drastically increased, accompanied by higher mortality rates. KS was also seen for the first time in the United States and Western Europe, clustering in HIV-infected men who have sex with men (MSM). At the time, the medical and scientific community suspected an infectious etiology for KS but could not confirm the pathogen. Confusing the picture was the fact that while KS was considered an AIDS-defining illness, it did not manifest in all patients with HIV/AIDS.

In 1994, Moore and Chang solved a critical piece of the mystery surrounding the origins of KS. Using representational difference analysis, the husband and wife team sequenced a previously described herpesvirus in a KS lesion from a patient with AIDS [5]. Using PCR testing, they subsequently confirmed the presence of HHV-8, now known as Kaposi Sarcoma herpesvirus or KSHV, in all four epidemiological subtypes of KS, including HIV-negative patients with the disease. This discovery established the critical role of KSHV in tumorigenesis [6]. Subsequent seropositivity surveys for KSHV in equatorial and South Africa, as well as MSM in the U.S. and Europe, showed disproportionately high rates of infection in these groups, which mirrored the prevalence of KS in these populations. Taken together, these molecular and epidemiological studies supported a cohesive narrative for the role of KSHV infection in KS. Elucidating the precise mechanism of viral oncogenesis would soon follow.

5.2.2 Epidemiology

The earliest reports of KS in the nineteenth century described lower extremity tumors in elderly Ashkenazi and Mediterranean men, which is now known as "classic KS." In the 1950s, reports of KS in Sub-Saharan Africa entered the literature, changing the epidemiological landscape of KS and our understanding of its clinical presentation. A government-funded push in the 1960s to document KS in African cancer registries highlighted its prevalence in parts of equatorial and South Africa [7]. Based on the data from these cancer registries, the incidence of KS in those regions at that time was 1 or 2 per 1000—compared to just 0.05 per 1000 in England.

In the 1980s, incidence rates of KS increased dramatically—up to 20-fold in some parts of Africa—as the AIDS epidemic took hold. A similar epidemiological shift was occurring across the Atlantic in the United States, particularly among the MSM population. The sudden appearance of KS in this subset of patients, the vast majority of whom were infected with HIV, signaled a possible infectious origin for KS. Incidence rates worldwide continued to climb in the 1980s and early-1990s as the number of HIV/AIDS cases skyrocketed. Prior to the advent of HAART, HIV-infected persons were at 2800-fold increased risk for developing KS compared to the general population [8].

The variability in cutaneous manifestations, patient demographics, and outcomes of KS observed in different populations called for a new classification system, which exists in its current form as (a) classic, (b) African or endemic, (c) AIDS-related, and (d) iatrogenic. In each epidemiologic group, KSHV is required for tumor development. Worldwide incidence rates for each type are difficult to estimate since cancer registries in Europe (where endemic, classic, and AIDS-related types overlap) do not differentiate between them.

Classic KS commonly affects middle-aged and elderly men of Jewish or Mediterranean descent, with the highest incidence rates reported in in Italy, Greece, and Iceland [9]. Immunosuppression is not a risk factor for classic KS, which favors the lower extremities and follows a benign course. Oropharyngeal or visceral involvement is exceedingly rare.

African or endemic KS also tends to affect men, although in some parts of Africa, the number of cases in men and women is roughly equal. Children are also affected. Violaceous nodules and plaques characteristic of KS tumors are the most common presenting feature across all age groups. In contrast to adults, lymphadenopathy is frequently seen in children and may be the sole presenting sign. Incidence rates for endemic KS have declined with improved access to HAART through international assistance programs [9].

KS is considered an AIDS-defining illness. The dramatic increase in KS cases in the 1980s coincided with the AIDS epidemic, and its subsequent decline in the 1990s mirrored the successful implementation of HAART. Despite this encouraging trend, AIDS-associated KS remains the most common malignancy in some African countries. While AIDS-associated KS disproportionately affects MSM in the United States and Western Europe, the incidence of KS in African men and women is roughly equal; prior to the AIDS epidemic, only 10–15% of KS cases were reported in women [10]. As in other KS subtypes, the cutaneous lesions of KS in HIV-infected persons typically present on the lower extremities, although oropharyngeal and/or visceral involvement is not uncommon. As in endemic KS, women are more likely than men to have lesions on the hard palate, while lymphadenopathy is disproportionately seen in children. Immune-reconstitution-inflammatory-syndrome KS (IRIS-KS) describes a small subset of patients who develop worsening KS after initiating HAART; the clinical presentation is similar to AIDS-associated KS, with the potential for both cutaneous and visceral involvement. IRIS-KS responds to systemic steroids [11].

5.2.3 Mechanisms of Carcinogenesis

Since its discovery in 1994, KSHV has been identified as the causative pathogen not just in KS, but also in two primary B-cell lymphoproliferative disorders, primary effusion lymphoma (PEL) and Castleman's disease. Epidemiological and molecular studies to date have established its critical role in all three diseases, while at the same time broadening our understanding of viral oncogenesis, which relies on an

interplay of cellular, genetic, and inflammatory factors to the drive the transformation of infected cells into neoplastic ones. The precise mechanism of how this occurs in KS lesions has yet to be fully elucidated—and indeed, varies based on the host's immune status and other factors—but has benefited from tremendous new insights into KSHV.

KSHV is a double-stranded herpesvirus with a large genome that contains over 85 genes, most of which have antigenic potential [12]. Unlike other herpesviruses, for which seroprevalance rates are high worldwide, KSHV is common in Africa and somewhat rare in the United States and Europe [13]. KSHV also demonstrates a clear predilection for HIV-infected and immunosuppressed patients; unsurprisingly, rates of KS are highest in these groups. That said, KSHV positivity is not sufficient for the development of KS, PEL, or Castleman's disease. While KS is a malignancy of endothelial cells, KSHV can infect other cell lines including monocytes and B-cells. Whether KSHV achieves its oncogenic potential or not depends on multiple factors, including activation of its viral proto-oncogenes, suppression of host cell tumor suppressors, evasion of the immune response, and alteration of the cell cycle.

The KSHV genome is encoded in an extra-chromosomal episome in the cell nucleus, which allows it to maintain replication during host cell division. KSHV has two phases of infection: latent and lytic. The virus replicates only during the lytic phase, which can be triggered by environmental factors, hypoxia, and co-infection with other viruses, as well as various cytokines in the host milieu. KHSV infection of endothelial cells, for example, induces expression of IL-6, which in turn increases KSHV viral load. KSHV also produces a viral homologue of IL-6, which promotes the Th-2 cell response while simultaneously interfering with the anti-tumor Th-1 cell response by reducing IFN-gamma production and preventing Th-1 cell differentiation [14]. Targeting another arm of the immune response, Cox2 is overexpressed in KS tumors and recruits pro-angiogenic factors such as PDGF and VEGF [15]. Through these mechanisms, KSHV propagates its own life cycle by promoting cell survival and enhancing angiogenic properties of infected endothelial cells. With genetic instability taking place in the host cell, likely as a result of this virally manipulated immune response, neoplastic transformation can take place. The degree of immune dysfunction in AIDS also plays a role in this process, as the survival and proliferation of KSHV depends in part on its ability to evade the immune response and immune-mediated apoptosis.

The other piece of the oncogenesis puzzle is the nuanced genetic machinery inherent in the KSHV genome. These viral proteins, which have largely been characterized by in-situ hybridization and immunochemical techniques, are the subjects of intense study. In 95% of KS cells, KSHV exists in its latent form. One protein expressed during this phase is the viral latent nuclear antigen LANA. LANA is thought to promote chronic KSHV infection by ensuring its survival in host daughter cells, but it may also promote activation of the lytic phase [16]. Other latent-phase proteins include FLIP, which induces IL-6 expression; and vCYC, which modulates the cell cycle [17, 18]. Kaposin and K15 are lytic-phase proteins that promote angiogenesis and mediate the immune response [19]. Clearly the balance of latent and lytic infection play a complimentary role that has yet to be fully eluci-

dated. In an effort to understand gene expression in KSHV-infected cells, the characterization of viral proteins not only broadens our understanding of oncogenesis but also points to potential therapeutic targets.

5.2.4 Controversies and Open Questions in the Field

By infecting endothelial cells, KSHV promotes angiogenesis and induces inflammation, which in turn leads to cellular proliferation and tumorigenesis in KS. However, significant questions about the pathophysiology of KSHV and its behavior in select patients remain at the forefront of scientific study. In murine models, for example, scientists have struggled to replicate the oncogenic events that take place in human KS lesions. Without a good animal model, efforts to develop anti-angiogenic treatments and other therapeutic targets for KS have posed challenges. As our understanding of KHSV and its pathogenesis in KS lesions continues to expand, one can hope that our therapeutic arsenal for this disease will do the same.

5.3 Merkel Cell Carcinoma and Merkel Cell Polyomavirus

5.3.1 Discovery

In 2008, 36 years after primary cutaneous neuroendocrine carcinoma was first described, Moore and Chang at the University of Pittsburgh —the same team to identify the oncogenic role of HHV8 in KS—characterized a definitive link between a small, double-stranded DNA polyomavirus and a rare neuroendocrine tumor, now known as Merkel cell carcinoma (MCC) [20]. For years, scientific study focused on the presumed but debatable relationship between MCC and its cell of origin, the Merkel cell. These investigations have benefitted from the advent of more sophisticated immunohistochemical stains and molecular techniques and continue to offer new insights into the pathogenesis of MCC. Likewise, our understanding of the so-called Merkel cell polyomavirus (MCPyV) continues to evolve.

The oncogenic potential of polyomaviruses was first described in 1953 by Gross et al. using murine models to study tumors with an infectious origin [21]. Characterizing the polyomavirus' role in human cancers, however, would come decades later, with the mainstream application of more advanced molecular techniques. Moore and Chang used digital transcriptome subtraction to identify a fusion transcript between a previously undescribed viral T antigen and a human tyrosine phosphatase, and from there sequenced a 5387-base-pair genome polyomavirus. The polyomavirus is a small, double-stranded DNA virus that encodes a variably spliced oncoprotein; in previous animal studies it had demonstrated an ability to integrate into the host genome, an erroneous lifecycle event that preceded tumor development.

Investigators used cDNA libraries from two MCC tumors to detect rare viral sequences, which confirmed integration of the MCPyV into the host genome. The viral sequences characterizing this particular human polyomavirus had been previously undescribed. Of note, there are three known genetic groups of polyomavirus; only one of these has been shown to infect humans. Closer study of the sequences in MCPyV revealed conserved features with other polyomavirus T antigens, particularly in the replication origin.

To further establish the connection between MCPyV and MCC, ten distinct MCC tumors were tested for MCPyV positivity using PCR testing. Two control groups were used for comparison. The first control group contained tissue samples from healthy 59 patients, while the second group had 25 patients. None carried a diagnosis of MCC. Southern blotting was performed to improve specificity. In eight of the ten MCC tumors, PCR testing was positive for MCPyV. None of the 59 patients in the first control group and only 4 of the 25 in the second group demonstrated positivity; the authors thus concluded that this newly described polyomavirus was definitively linked to MCC. Southern blot techniques provided evidence that MCPyV infection and integration into the genome preceded the clonal expansion of tumor cells.

5.3.2 Epidemiology

Approximately 1600 cases of MCC are diagnosed in the U.S. each year, which reflects a steady increase in incidence rates over the past three decades [22]. Part of this rise is due to increased reporting. MCC is a disease of the elderly, and with people in the U.S., New Zealand, and Europe living longer—where most cases are reported—it is no surprise that MCC is on the rise. Increasing incidence rates have also been attributed to the advent of the cytokeratin-20 stain in 1992, which led to more accurate and reliable diagnosis. Diagnostic techniques have continued to evolve since that time and largely rest on the histopathologic assessment given MCC's non-specific clinical presentation, although no standard immunohistochemical panel for MCC exists.

The overall-age adjusted worldwide incidence rate for MCC based on the most recent population studies from the Surveillance, Epidemiology, and End Results (SEER) program in the United States is 0.79 cases per 100,000 people per year [22]. Cancer registries in Europe and New Zealand report similar incidence rates, which in part reflects their significant Caucasian population. MCC is approximately 25-times more common in white persons compared to other ethnic groups, although it has been reported in Pacific Islander, American Indian, Asian, and black patients [23]. Incidence rates in geographic areas dominated by these ethnic groups have not been calculated due to the scarcity of cases reported there.

MCC most commonly affects elderly white males with a history of abundant sun exposure. The incidence rate among whites is higher in Hawaii than the mainland U.S, for example, and appears to correlate with high UV-indices. MCC affects

nearly twice as many men as women, and it almost always occurs in patients over the age of 65. Head and neck tumors are most common, but MCC has also been reported on the trunk, extremities, and mucosal surfaces.

5.3.3 Mechanisms of Carcinogenesis

The pathogenesis of MCC is multifactorial and complex. Genetic, molecular, and environmental factors are all at play in tumorigenesis; the association of MCC with UV-radiation, for example, has been well-described in numerous population-based studies [24]. More recently, our understanding of the pathogenesis of MCC has changed drastically with new insights into viral oncogenesis, as 80% of MCC are polyomavirus-positive. The differences between polyomavirus-positive and negative tumors are significant. Tumor cells in polyomavirus-negative MCC, for instance, demonstrate a prominent UV-signature mutational pattern; interestingly, this signature has not been observed in polyomavirus-positive patients [25, 26].

There are other notable differences, as well. On a molecular level, MCPyV-positive tumors are characterized by Rb1 gene inactivation and mTOR pathway changes, absent p53 mutations, and fewer overall mutations; one study showed a 20-fold difference in the number of mutations compared to MCPyV-negative tumors [25]. MCPyV-negative tumors demonstrate increased cellular atypia, high Ki-67 positivity, truncating mutations in Rb genes, p53 and HRAS mutations, as well as loss-of-function mutations in PRUNE2 and NOTCH genes [26]. The tumor histology also differs between the two; MCPyV-negative tumors have more irregular nuclei than MCPyV-positive MCC.

The survival benefit of viral infectivity has yet to be fully characterized, but based on the largest epidemiologic studies conducted to date, the presence of polyomavirus in tumor cells is considered a positive prognostic indicator. There are several potential reasons for this survival benefit. In contrast to the "typical" MCC presentation, MCPyV-positive tumors tend to present on the trunk and extremity, as opposed to the head and neck. Additionally, patients with MCPyV-positive tumors are more likely than their MCPyV-negative counterparts to have localized disease at the time of diagnosis [27]. Women are more likely to have MCPyV-positive tumors than men, which is notable because female gender is considered a positive prognostic indicator in MCC [23]. Lastly, as discussed above, the mechanisms of carcinogenesis are affected by viral status; in MCPyV-positive tumors that fail to evade the immune response, for instance, the cure rate *in vitro* is essentially 100%. This has led to the recent development of immunotherapy as a therapeutic strategy for MCC.

What remains to be fully characterized is the specific mechanism of tumorigenesis in MCPyV-positive tumors, although great strides toward this end have been made recently. Building upon Moore and Chang's initial proposition that MCPyV achieved its oncogenic potential in vulnerable cells through a combination of UV radiation exposure, expression of specific non-self T antigens, and evasion of the

immune response, several studies have delved more deeply into this sequence of events [28, 29].

As noted above, MCPyV-positive MCC lacks a mutational UV-signature, which not only contrasts with its virus-negative counterpart but also highlights other molecular events at play. In MCC tumor cells, MCPyV integrates into the genome at a non-specific binding site and expresses two putative oncoproteins, the large T-antigen and small T-antigen. The truncated domain of the large T antigen may play a role in shifting the virus' natural behavior from that of replication and virion release to clonal integration and tumorigenesis. Acting on different molecular pathways, these viral oncoproteins are capable of modulating the cell cycle and inducing unregulated cellular proliferation. In MCPyV-positive tumors, the binding of a truncated large T-antigen inactivates the tumor suppressor Rb, whereas Rb itself is mutated in virus-negative tumors [28, 29]. Downstream effects of large T-antigen expression include protein dysregulation that leads to the accumulation of oncoproteins in the cell nucleus, such as cyclin-E, c-Jun, and mTOR [30]. Small T-antigen is thought to initiate tumorigenesis through coexpression of the cell fate-determinant atonal bHLH transcription factor 1 (ATOH1) [31]. These cellular events promote the survival of the tumor cell and handicap the immune system's programmed response to viral infection and dysregulated growth.

More generally, the vast majority of MCC tumors downregulate the expression of MHC class I, which allows MCPyV-derived peptides to evade detection by CD8 T cells. Additionally, vascular E-selectin expression is reduced in many MCC tumors, both MCPyV-positive and negative, which hampers lymphocytic migration and inhibits a coordinated immune response [32]. Several studies have shown that alterations to T-antigen expression and its targets reduce or in some cases completely negate its oncogenic potential; these discoveries have shaped the landscape of new immunomodulator therapies in clinical trials [33, 34].

5.3.4 Controversies and Open Questions in the Field

Polyomaviruses are not new, nor are they especially uncommon; up to 80% of the general population is infected with MCPyV [35, 36]. How and why this common virus selectively targets certain cells and drives the development of such an aggressive tumor in a relatively small number of people remains under study. Clonal integration of MCPyV into the host genome, for example, does not occur in non-lesional skin. The susceptibility of select cells to MCC is likely due to a combination of mutations induced by UV radiation and immune dysregulation, but the precise mechanism of viral oncogenesis has not yet come into clear focus. Given the number of significant discoveries in the last 10 years, there is much to be excited about in this field of study.

In regards to management, serologies measuring T-antigen antibody levels have been developed as a surveillance tool for MCPyV-positive patients, but have yet to achieve widespread use in clinical practice. Immunotherapies such as PD-L1 and

IL-2 inhibitors are in clinical trials but not yet FDA-approved for MCC. With enhanced understanding of tumorigenesis in MCPyV-positive MCC, the development of more targeted therapeutic agents are likely to enter the mainstream.

5.4 Cutaneous Squamous Cell Carcinoma and Human Papillomavirus

5.4.1 Discovery

The carcinogenic potential of HPV in cSCC was originally noted in patients with the rare hereditary genodermatosis epidermodysplasia verruciformis (EV), described in 1922 by Lewandowsky and Lutz. These patients present with widespread verrucous lesions in sun-exposed areas, some of which undergo malignant transformation [37]. The identification of HPV in these lesions was among the first evidence that cSCC could be linked to HPV [38–40]. These EV-type HPV were later classified as β-genus HPV, and have tropism to cutaneous epithelium. More attention has been paid to the mucosal α-genus HPV; the identification of low- vs. high-risk αHPV in genital condyloma and cervical carcinoma established the relationships between the two and broadened our understanding of αHPV's oncogenic potential in mucosal epithelium [41]. Multiple studies have attempted to demonstrate parallel oncogenic mechanisms for βHPV in cutaneous epithelium, but there are significant differences that are still incompletely understood.

5.4.2 Epidemiology

The incidence of cSCC is on the rise worldwide, particularly among people of European descent [42–44]. cSCC is typically described as a tumor occurring on sun-exposed areas, and indeed, ultraviolet radiation is the primary risk factor for cSCC. In at least some cases of cSCC, actinic keratoses (AK) undergo malignant transformation years or even decades after their initial presentation. The annual risk of any particular AK lesion evolving into cSCC is unknown, although a recent systematic review suggests that the number is exceedingly low, ranging from 0% to 0.53% per lesion-year [45]. Incidence rates vary widely across geographic areas, due at least in part to differences in ultraviolet radiation, but in the U.S., overall, there are approximately 200 cases of cSCC per 100,000 person-years [46]. cSCC is more common in men than women, and its incidence increases with age. While the head and neck are the favored sites of involvement, cSCC can occur on anywhere on the body, including the genitals and feet [47].

Aside from UV radiation, other risk factors for cSCC include fair skin, iatrogenic and systemic immunosuppression, arsenic, and other chemical exposures [48]. HPV has been considered a potential oncogenic agent, but efforts to define causal link

have been inconclusive. There have been many efforts over the years to measure the prevalence of HPV in cSCC lesions, but studies have shown wide variation in HPV prevalence. Much of the variance appears to be due to differences in patient population and immune status, tissue source (including skin biopsy, hair pluck, blood), and typing method (including serology, DNA PCR, ELISA or high throughput sequencing) [49]. Two recent meta-analyses of studies investigating the association between HPV and cSCC ultimately concluded that there is at least a solid epidemiologic association between HPV and cSCC, reporting that tumor tissue had 1.4- to 3.4-fold odds of carrying HPV compared to normal tissue [50, 51], and that tumors immunocompromised subjects were three times more likely to carry HPV than tumors from immunocompetent subjects [51]. These data support ongoing efforts to understand the molecular or immunologic mechanism of carcinogenesis.

Serologic testing has been similarly inconclusive. Most studies have shown no relationship between HPV seropositivity and cSCC, although this analysis is limited by several factors, including delays in seroconversion, variations in HPV types, and antibody cross-reactivity [50]. One case report showed a dramatic decrease in the development of cSCC in two immunocompetent patients with multiple tumors who received the quadrivalent HPV-vaccination, which may point to a therapeutic target but requires additional study [52].

5.4.3 Mechanisms of Carcinogenesis

Papillomaviruses are small, double-stranded DNA viruses with epithelial tropism. There are over 150 types of papillomaviridae; HPV is subdivided into the five genera: alpha, beta, gamma, mu and nu [53, 54]. αHPV mainly infects mucosal epithelium, and its oncogenic properties are well characterized with respect to cervical and head and neck SCC. Low-risk αHPV are associated with genital warts, while high-risk αHPV can transform mucosal keratinocytes and immortalize their cell cycle, leading to SCC [55, 56]. On a basic molecular level, E6 binds to the p53 tumor suppression gene and marks it for proteolytic degradation, while E7 binds to and inactivates the retinoblastoma tumor suppressor gene [57]. Taken together, these events disrupt the normal cell cycle, which promotes unregulated cellular proliferation (reviewed in [58]).

Both βHPV and γHPV infect cutaneous epithelia, and a number of HPV types belonging to these genera have been found in normal skin, benign lesions, and cSCC. βHPV are considered carcinogenic for cSCC in patients with EV, but there has not been sufficient data to support an oncogenic claim in cSCC in the general population [59]. The majority of translational studies have assessed prevalence of HPV DNA but not viral loads; one study indicated a higher viral load in precancerous dysplastic actinic keratosis compared to invasive cSCC [60], while another demonstrated that the viral loads in cSCC are fewer than 1 copy per 100 cells [61]. While αHPV integrates into the host genome and directly drives oncogenesis through viral protein transcription, βHPV does not [61–63]. This suggests that any

role for βHPV in cutaneous squamous carcinogenesis occurs through a "hit and run" mechanism in initiation, and may occur synergistically with UVR [64]. In normal skin, UVR causes signature mutations that trigger the activation of the tumor suppressor p53, which protects the cell by recruiting DNA repair proteins, arresting the cell cycle, and initiating apoptosis. In βHPV-infected cells, E6 cannot directly inhibit or degrade p53—as has been observed in αHPV—but it may interfere with the downstream effect of p53. In βHPV 5 and 8, E6 has been shown to manipulate the cellular machinery in several ways: it blocks the pro-apoptotic Bak signaling pathway following UV damage; induces EP300/CREBBP degradation, which blocks the phosphorylation of p53; inhibits Notch signaling by binding the MAML-1 cofactor, which impairs the cell's innate machinery to suppress tumor development; and inhibits TGF-β signaling, which plays a complex role in carcinogenesis [65].

Until recently, our ability to understand these pathways *in vivo*, particularly in the context of transient or hit-and-run mechanisms, was limited by the lack of a reliable animal model. Recent efforts to study the skin of the multimammate rat Mastomys coucha, which is infected by Mastomys natalensis papillomavirus (MnPV), have opened additional avenues for understanding the role of HPV in cSCC [66, 67].

5.4.4 Controversies and Open Questions in the Field

The strong epidemiologic link between βHPV and cSCC supports ongoing efforts to clarify a viral mechanism for oncogenesis. Classically, tumors caused by DNA oncoviruses require ongoing direct viral protein expression to maintain cancer replication, so-called "oncogene addiction." βHPV does not exhibit this direct oncogenic capacity, nor does it integrate into the genome to disrupt host tumor suppressors via indirect oncogenesis. How HPV interacts with UV radiation and host immunity to stimulate tumor initiation is a fascinating topic of ongoing research.

5.5 Bacterial Skin Commensals and Skin Cancers

5.5.1 Discovery

While the oncogenic role of viruses have been well-established and fairly understood in certain skin cancers, the potential contribution of bacteria in cutaneous carcinogenesis is yet to be elucidated. Colonization of skin by bacteria begins immediately after birth to include several resident commensal bacteria and transient potentially pathogenic bacteria [68]. The type and number of bacteria (and other microorganisms) is determined by numerous factors including host characteristics such as age (infancy, puberty), sex (pregnancy), ethnicity, hygienic routine, lifestyle exposures, topical medication and/or cosmetic use and systemic diseases [68–70]; genetic factors such as primary immunodeficiency syndromes [71]; environmental

parameters such as geographic location, humidity and crowding [72, 73]; and specific cutaneous factors such as anatomic site, moisture levels, pH and skin diseases such as chronic dermatitides, ulcer, abscess, etc. [2, 70, 71, 74].

5.5.2 Epidemiology

While numerous varieties of bacteria reside on the skin, *Propionibacterium*, S*taphylococcus*, *Micrococcus* and *Corynebacterium species* are the most common skin commensal bacterial genera in healthy skin [75, 76]. Of these gram-positive bacteria, coagulase negative *Staphylococcus epidermidis* is most common in interfollicular epidermis, while the *Propionibacterium acnes* primarily colonizes the pilosebaceous units [77, 78]. *S. epidermidis* strains produce a variety of antimicrobial peptides [79], including bacteriocins, while *P. acnes* produce lipases [80], which retard growth of transient pathogenic bacteria such as Group A Streptococcus (GAS) and *S. aureus*, among others [77]. In addition, *S. epidermidis* also interact with dermal dendritic cells and induce influx of IL-17A-positive cytotoxic T-cells [81]. Thus, these bacteria contribute to cutaneous immunity [4, 74]. However, *S. epidermidis* and *P. acnes* can also become pathogenic, causing nosocomial infections and acne, respectively, under permissive conditions. Patients with primary immunodeficiencies such as Wiskott-Aldrich syndrome, hyper IgE syndrome, etc. have altered skin microbiome, characterized by decreased biodiversity and relative abundance of *S. aureus*, *Clostridium* sp., *Corynebacterium* sp. and *Serratia marcescens* [71].

To date, no bacterial pathogen has been causally associated with development of skin cancers. However, patients with certain chronic skin diseases may have altered skin microbiome with colonization of certain pathogenic bacteria and may have different risks for developing cutaneous malignancies, compared to the general population.

For instance, certain strains of *S. aureus*, such as the clonal complex have been implicated in playing a role in the pathogenesis of atopic dermatitis [82]. A meta-analysis of 95 studies revealed a 70% colonization rate, with increased incidence in lesional skin and higher severity of disease [83]. A population-based case control study revealed that female patients with atopic dermatitis have reduced risk for developing basal cell carcinoma and cSCC, whereas male patients had an increased risk for developing cSCC [84]. Increased risks for developing cSCC were also observed in a retrospective case control study, in both men and women with atopic dermatitis [85]. Lesional skin of psoriasis patients also has altered skin microbiome, with increased abundance of *Firmicutes*-associated and *Actinobacteria*-associated microbiota [86]. A recent study suggests increased prevalence of skin cancer among psoriasis patients [87].

Hidradenitis suppurativa (HS), a chronic skin disease characterized by development of recurrent and progressive suppurative nodules and sinus tracts with extensive scarring of the intertriginous areas, may be complicated by development of cSCC [88]. Peptide nucleic acid-fluorescence in situ hybridization analysis revealed decreased

bacterial load and decreased biofilm formation in the unaffected axillary skin of HS patients compared to normal subjects [89]. However, next-generation sequencing of 16S and 18S ribosomal RNA revealed alteration of follicular bacteria in HS patients, with relative decrease in *Propionibacterium* sp., including *P. acnes* and abundance of *Porphyromonas* sp. and *Peptoniphilus* sp. in lesional skin among others [90].

5.5.3 Role in Carcinogenesis

While studying coagulase-negative *Staphylococcus* sp. isolated from healthy human skin, Nakatsuji et al. identified that a strain of *S. epidermidis* produced a molecule with bactericidal activity against GAS [91]. This molecule, 6-N-hydroxyaminopurine (6-HAP) had a structure similar to adenine and was capable of inhibiting DNA polymerase in several tumor lines, including B16F10 (melanoma) and Pam212 (SCC), but not in NHEK (non-transformed human epidermal keratinocyte) cell lines. This tumor-specificinhibitory activity of 6-HAP was due to high level expression of mitochondrial amidoxime reducing components in non-transformed cells. Intravenous administration of 6-HAP reduced progression of B16F10-inoculated tumors in C57BL6 mice, while colonization of skin by *S. epidermidis* strains producing 6-HAP reduced the number of UV-induced tumors in Skh-1 mice. Analysis of the human skin microbiome metagenomic data revealed 6-HAP producing strains of S. epidermis from the skin of various body sites in normal healthy individuals. While there is much to be understood regarding the association of 6-HAP producing bacteria and cancer risk, this study suggests that certain bacteria can directly affect cutaneous carcinogenesis.

5.5.4 Controversies and Open Questions in the Field

Our current understanding regarding the contribution of cutaneous bacteria to development of skin cancer is limited. While rare associations have been reported, a direct causative role has not been established. Whether altered skin microbiome plays a permissive role in cutaneous disease is yet to be proven.

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Chapter 6
The Role of the Human Virome in Hematologic Malignancies

Rosemary Rochford, Carrie B. Coleman, and Bradley Haverkos

Abstract The focus of this Chapter will be on the viruses that can persistently infect humans becoming permanent members of the human virome. These viruses include Epstein-Barr virus (EBV), Kaposi's sarcoma herpes virus (KSHV), hepatitis C virus (HCV) and human T-cell leukemia virus (HTLV)-1. EBV, KSHV and HTLV-1 establish latent infections in lymphocytes that cannot be eradicated while HCV leads to chronic infection that can be ultimately cured with anti-viral drugs. The hematologic malignancies associated with these viral infections include B, T and natural killer (NK) cell lymphomas and adult-T cell leukemia. A challenge in understanding the etiology of the viral-associated hematologic malignancies is the relative ubiquity of the viruses within the human population in contrast to the rarity of the associated malignancies. Nonetheless, it is clear that these members of our human virome contribute to a substantial burden of hematologic malignancy.

Keywords Virome · EBV · KSHV · HCV · HTLV-1 · Hematologic malignancy

6.1 Introduction

As we begin to understand more about the human microbiome and its role in health and disease, attention has turned to understanding the virome. The virome includes not only viruses that infect human cells, but also endogenous retroviruses that have colonized the human genome and viruses that infect the bacteria that make up the microbiome. The focus of this Chapter will be on the viruses that persistently infect humans, becoming life long companions so to speak. These viruses include Epstein-Barr virus (EBV), Kaposi's sarcoma herpes virus (KSHV), hepatitis C virus (HCV)

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Table 6.1 Viral hematologic malignancies

Virus	Hematologic malignancy	Lymphoproliferative disorders	Viral oncogenes
EBV	Burkitt lymphoma, Hodgkin lymphoma, diffuse large B cell lymphoma, plasmablastic lymphoma, primary effusion lymphoma, extra-nodal NK/T lymphoma, angioimmunoblastic T-cell lymphoma, hydroa vacciniforme-like lymphoma	systemic T-cell lymphoproliferative disease of childhood, immunodeficiency linked B cell lymphoproliferative disease	EBNA-1, EBNA-2, EBNA-3a, EBNA-3c, LMP-1 LMP-2
KSHV	Primary effusion lymphoma	MulticentricCastleman's disease	LANA, vFLIP, vCYC
HTLV-1	Adult T-cell leukemia	None known	Tax, HBZ
HCV	Diffuse large B cell lymphoma (DLBCL), marginal zone lymphomas, lymphoplasmacytic lymphomas	Mixed cryoglobulinemia	None known

and human T-cell leukemia virus (HTLV)-1. While all of these viruses can cause non-hematologic diseases, they are also associated with a number of hematologic malignancies (Table 6.1). The challenge with understanding the etiology of these malignancies is the relative ubiquity of their associated viruses which contrasts with the rarity of these malignancies. In this chapter, we will describe the biology of the persistent viruses that are part of our virome, the hematologic malignancies they are associated with and finally, potential mechanisms that drive persistent viral infections into disease.

6.2 Hematologic Malignancies

Hematologic malignancies derive from cells of the immune system and can be of either myeloid or lymphoid origin. While there are some malignancies that are derived from cells of myeloid origin, the cancers associated with viruses that are part of our virome derive primarily from cells with a lymphoid origin, e.g. B cells, T cells, and natural killer (NK) cells. These malignancies can be classified as either lymphomas or leukemias. Lymphomas are further classified as Hodgkin or non-Hodgkin lymphomas (NHL). According to the World Health Organization (WHO) criteria for classifying lymphomas, there are >60 subtypes of lymphomas [1]. The viral-associated lymphomas include the B cell derived malignancies such as Burkitt's lymphoma, diffuse large B cell lymphoma, primary effusion lymphomas, plasmablastic lymphomas, T and NK cell lymphomas, and a spectrum of lymphomas arising in setting of immunosuppression (e.g. post-transplant lymphoproliferative disorders, HIV). While lymphomas typically are found in lymph nodes (but not always), leukemias are generally found as an expansion of lymphocytes or myeloid

cells in the blood. Of the leukemias, only adult T-cell leukemia has a clear association with a viral infection, i.e. HTLV-1.

Because T and B cells have to undergo somatic gene rearrangement to generate T cell receptors and B cell receptors, respectively, as well as somatic hypermutation in the case of B cells, the machinery needed to alter the genome is activated in these cells. This is thought to increase their susceptibility to malignant transformation. These cells undergo repeated division throughout the life of the host, further increasing their vulnerability to additional genetic alterations. Finally, as we will describe below, the viruses that infect these cells encode oncogenes creating additional opportunities for transformation.

6.3 Viruses Associated with Hematologic Malignancies

6.3.1 EBV

EBV, also called human herpesvirus 4 (HHV-4), is a member of the gammaherpesvirus family and is a double stranded enveloped DNA virus. The viral genome is ~172 kilobase pairs (kbp) and encodes genes necessary for viral replication and for viral latency. There are two strains of EBV, EBV type 1 and type 2 that exhibit both genotypic and phenotypic differences [2, 3]. EBV type 1 is the predominant strain world-wide and is the most widely studied. EBV type 2 is more common in Africa and less frequently in Western and Asian populations [2, 4]. Greater than 90% of the global population is infected with EBV [5, 6] making it one of the most successful viruses and a prominent member of the human virome.

EBV is a strict human pathogen. Oral transmission through direct contact with infectious saliva is considered to be the primary route of transmission. There are two peaks of EBV infection as measured by seroconversion, age 2–4 years and 15 years [7]. In sub-Saharan Africa, most children are infected with EBV by 2 years of age [8, 9] with some infected at less than 6 months of age [10].

EBV can infect B cells, T cells, and NK cells along with epithelial cells. Life long persistence of the virus is thought to be in B cells [11], but recent data suggests that T cells might also serve as a reservoir for EBV type 2 [12]. EBV is unique among viruses in that, in contrast to most viruses that establish lytic infection a priori, primary infection of B cells results in establishment of a latent viral infection [13]. In culture, this leads to the immortalization of B cells and expression of all the latency genes [14, 15].

The study of EBV latency has led to a defining paradigm of EBV biology, e.g. the virus' ability to establish different latency programs in normal and malignant B cells. EBV encodes nine latent proteins: latent membrane protein (LMP)-1, LMP-2a, LMP-2b, Epstein-Barr nuclear antigen (EBNA)-1, EBNA-2, EBNA3a, EBNA3b, EBNA3c, and EBNA-leader protein (LP). The latency program of EBV in health mirrors the latency program found in EBV-associated malignancies [16]. For

example endemic Burkitt lymphoma (BL) expresses only EBNA-1, diffuse large B cell lymphoma of the elderly and Hodgkin's disease expresses EBNA-1, LMP-1 and LMP-2 and immunoblastic lymphoma expresses all the latent proteins [17]. While the majority of the cells within the EBV-positive hematologic malignancies are typically latently infected, lytic transcripts and proteins are sometimes found [18]. The contribution of viral lytic cycle proteins to malignancy remains unknown but studies in humanized mouse model implicate at least the EBV immediate early protein, Zta, in lymphomagenesis [19]. In addition, small noncoding (nc) RNAs are also expressed during latency and in EBV-lymphomas including the EBV encoded small RNA (EBER) 1 and 2, and up to 50 microRNA's [20]. Because the EBERs are highly expressed in infected cells, in situ hybridization to detect EBERs has been widely used clinically to detect EBV in lymphoma tissues [21]. Beyond their practical role in pathology, there is also indication that EBERs modulate host cell function and contribute to malignancy [22, 23].

EBV has been classified as Class I carcinogen by the International Agency for Cancer Research [24]. When you examine the list of EBV-associated hematologic malignancies, the variety is quite striking. EBV is associated with the B cell lymphoproliferative diseases found in immunodeficient hosts, as well as the following lymphomas: Burkitt lymphoma, Hodgkin lymphoma, diffuse large B cell lymphoma, plasmablastic lymphoma, and primary effusion lymphoma. In addition to B cell lymphomas, EBV is also found in extra-nodal NK/T lymphoma, angioimmunoblastic T-cell lymphoma, hydroa vacciniforme-like lymphoma and systemic T-cell lymphoproliferative disease of childhood [25]. A unique feature of EBV malignancies is the striking geographic prevalence of some types of malignancy. For example, endemic BL in sub-Saharan Africa has a clear link to *P. falciparum* malaria [26, 27] while T cell lymphomas are more prevalent in Asia [28].

The unique geographic and age prevalence of EBV-associated hematologic malignancies points towards the fact that EBV in most cases is likely necessary but not sufficient to drive lymphomagenesis. That said, extensive molecular and functional analysis of EBV latent proteins points towards clear roles for the viral proteins in driving lymphomagenesis. Of the nine EBV latent proteins, EBNA-1, EBNA-2, EBNA-3a, EBNA-3c, LMP-1 and LMP-2 have been shown to be essential for transformation of B cells [29]. While a discussion of the molecular studies of EBV latent proteins function is beyond the scope of this chapter, readers can refer to several recent comprehensive reviews [29–31].

6.4 KSHV

KSHV (also known as human herpesvirus-8), like EBV, is a human gammaherpesvirus and belongs to the subgroup gamma-2 herpesvirus. KSHV is a double-stranded enveloped DNA virus with a genome of ~160 kbp. The virus was discovered in 1994 by Chang and colleagues [32] in attempt to discover if there was an infectious cause

of Kaposi's sarcoma. KSHV shares many similarities with EBV including transmission through saliva [33] and life long latency reservoir in B cells [34]. However, KSHV has a much more limited worldwide distribution than EBV with geographic variability in it's distribution. In Africa, there is the so-called "KSHV belt" with greater than 50% KSHV seroprevalence [35, 36], the Mediterranean region has between 10% and 30% seroprevalence, while in northern European and USA, the seroprevalence is less 10% [37].

Infection in endemic countries occurs in children with a peak age of seroconversion around 6 years [38]. Risk of infection in childhood increases if the mother is also infected [39]. Sexual transmission in the context of the HIV epidemic was thought to increase the prevalence of this infection but whether KSHV is transmitted through semen remains controversial [40]. The current consensus is that the primary mode of KSHV transmission is saliva [41].

KSHV establishes both a latent and lytic infection. During latency in B cells, several viral proteins are expressed including latency associated nuclear antigen (LANA), and K1 as well as three cellular gene homologues, viral(v) FLIP, vIL6 and vCyclin, along with viral microRNAs [42]. While it is clear that the virus establishes life-long latency in B cells [43, 44], early attempts to infect B cells *ex vivo* were not successful limiting the understanding of KSHV pathogenesis to infection of endothelial cells and by analogy to B cells. Subsequently, it was found that activation of B cells prior to infection resulted in susceptibility to KSHV infection [45] and that KSHV targets a subset of tonsillar B cells [46].

KSHV is the causative agent of two B cell diseases: primary effusion lymphoma (PEL) and multicentric Castleman's disease (MCD), a B cell lymphoproliferative disease [47]. PEL is very rare and typically found only in those with underlying immunodeficiency primarily due to HIV [48]. PEL occurs in pericardial, pleural or peritoneal spaces. PEL cells are often co-infected with EBV [49] raising the question of whether these pathogens interact synergistically to promote lymphomagenesis [50]. MCD, while not a true malignancy, is a risk factor for development of plasmablastic lymphoma [51].

LANA is the only viral protein detected in all KSHV tumors [52] leading to an intense focus on LANA function. Several lines of evidence point to LANA's oncogenic capacity including the multifunctional nature of the protein as demonstrated in numerous studies [53]. A compelling case for LANA's oncogenic potential is from studies using transgenic mice; expression of LANA resulted in both B-cell hyperplasia and a slow onset of B cell lymphomas in a subset of older mice [54]. Two other proteins are also consistently detected in KSHV latently infected cells: vCYC and vFLIP [55]. Transgenic mice that express vFLIP generated tumors similar to PEL suggesting a role for this protein in lymphomagenesis [56]. Clues to the role of vCYC in lymphomagenesis come from studies where vCYC transgenic mice develop lymphomas [57]. Of note, this is only when the tumor suppressor protein p53 is deficient, highlighting the complex nature of oncogenesis and the requirement for multiple factors to drive lymphomagenesis.

6.5 Hepatitis C Virus

HCV was first described in 1989 [58] and is a member of the flavi-virus family. HCV is an enveloped single stranded positive RNA virus with a genome of only 9.6 kb. Following viral entry into hepatocytes, HCV replicates in the cytoplasm [59]. The virus encodes a large polyprotein that is cleaved to yield 3 structural proteins (core, E1, and E2) and 7 non-structural proteins (p7, NS2, NS3, NS4A, NS4B, NS5A and NS5B) [60]. There are no known oncogenes encoded by HCV.

Like many other small RNA viruses, HCV exhibits significant genetic heterogeneity. There are at least six major genotypes of HCV, with varying prevalence depending on geographic location [61]. There is no known disease association with a particular genotype. Upwards of 80 million people world-wide are chronically infected with HCV [62]. The geographic prevalence of HCV varies with China, Pakistan, Egypt and Nigeria having the highest prevalence and a significantly lower prevalence is observed in higher income countries [62]. HCV is transmitted to neonates through vertical transmission from infected mothers [63]. Transmission among adults is through sexual contact, sharing of contaminated needles, and iatrogenic [64]. Primary infection with HCV is generally asymptomatic. Following primary infection, the viral infection can be spontaneously cleared or establish a life-long chronic infection with ongoing viremia [65]. This is unlike the other viruses associated with hematologic malignancies which establish latent infections in their human host.

There is no doubt that HCV is a hepatotropic virus. Infection of lymphocytes has been more controversial. Both positive and negative strand HCV RNAs were detected in PBMC of chronically infected patients [66, 67]; the presence of the negative strand RNA suggests ongoing viral replication in lymphocytes. However, in follow-up studies, B cells were non-permissive for HCV infection in one study [68] and HCV infected B cells while not productively infected, promoted trans-infection of hepatocytes in a second study [69]. More recently, HCV variants were identified that had viral envelope glycoproteins with distinct lymphotropism as compared to other isolated variants from the same chronically infected patient that had hepatic tropism [70]. The presence of dual HCV variants within the same patient is intriguing and points to a dynamic interaction between lymphotrophic and hepatotropic HCV strains within the host. A recent study has found that CD86 (B7.2) is a co-receptor for lymphotropic variants of HCV on B cells [71] providing more credence that HCV infection of B cells is part of the biology of this virus.

Diffuse large B cell lymphoma (DLBCL) is the most common lymphoma subtype occurring with HCV infection in European cases [72]. Evaluation of a larger population cohort found that HCV is also associated with marginal zone lymphomas and lymphoplasmacytic lymphomas [73–75]. The incidence of HCV-associated NHL is higher in regions where the incidence of underlying HCV infection is high and likely represents up to 10% of NHL cases [76]. HCV is also associated with mixed cryoglobulinemia, a low grade B cell clonal lymphoproliferative disorder and a possible precursor to malignant B cells [74].

Beyond the epidemiologic associations of HCV with NHL, a stronger case for HCV as a cause of NHL came from a seminal study in 2002 [77]. Patients that had splenic lymphoma with or without concomitant HCV infection were given interferon therapy for treatment of HCV. Lymphoma regression occurred only in those patients that were HCV positive and had a sustained virologic response to the antiviral treatment. Subsequent studies have observed lymphoma regression in HCV+ splenic marginal zone lymphoma patients using only anti-virals [78–82].

In the absence of an oncogene, three possible mechanisms have been proposed linking HCV infection to NHL [83]. The first possible mechanism is chronic antigen stimulation of B cells via binding of HCV proteins to cognate antigen receptors on B cells. A second mechanism would be through binding of the viral E2 glycoprotein to the CD81 molecule on the surface of B cells driving polyclonal activation of naïve B cells. CD81 has been shown to be a high affinity receptor for HCV-E2 [84]. Either of these two mechanisms would results in chronic B cell stimulation, a consequence of which could be down-stream accumulation of genetic changes in the B cells. A final possible mechanism is through direct infection of B cells. With the more recent studies showing a lymphotropic variant of HCV [71], this possibility is gaining greater credence. However, downstream effects of persistent HCV infection on B cells are unknown.

6.6 HTLV-1

HTLV-1 is a delta RNA retrovirus with a single stranded RNA genome of 9 kbp and was isolated in 1980 [85]. Similar to other retroviruses, HTLV-1 integrates as a provirus in the host genome. HTLV-1 is transmitted through exposure to bodily fluids including breast milk, semen and infected blood products ([86–88]. HTLV-1 establishes a life-long infection in $CD4^+$ T-cells as well as $CD8^+$ T cells and dendritic cells [89].

Carriers of HTLV-1 infection are found world-wide with an estimated ten million HTLV-1 infected people [90]. Several regions have high endemicity for HTLV-1 infection including Japan, the Caribbean and South America, and sub-Saharan Africa [91]. HTLV-1 infection causes adult-T-cell leukemia (ATL). The cancer was first described in Japan in the 1970s [92]. ATL, as the name implies, is a disease that occurs in adults, typically several decades following primary infection. Less than 8% of those infected with HTLV-1 will go on to develop ATL with men having a higher risk (4.5–7.3%) than women (2.1–3.8%) [93]. There are four clinical subtypes of ATL: acute, lymphoma, chronic and smoldering [94].

HTLV-1 encodes four proteins (e.g. gag, pro, pol and env) essential for viral replication. In addition, through complex splicing, several regulatory proteins are also generated from the relatively small genome. These include Tax, Rex, HBZ (also known as bZIP), p12, p13 and p30 [95]. Of these, Tax and HBZ are thought to be the key drivers of oncogenesis [96, 97]. A puzzle early on was that although the epidemiologic data was strong that HTLV-1 infection was linked to ATL [93],

detection of Tax in leukemic cells was infrequent [96]. More recently, HBZ transcripts were detected at low levels in HTLV-1 infected cells suggesting a critical role for the HBZ protein in viral oncogenesis [97]. HBZ is a transcriptional transactivator and promotes cell proliferation [97]. Tax binds to DNA and is also a transcriptional transactivator [98]. Tax is an oncoprotein based on classic definitions, e.g. immortalizes cells in vitro, can stimulate colony formation in soft agar and Tax expressing cells can generate tumors following xenoengraftment in immunodeficient mice [99, 100]. In regards to ATL etiology, Tax is thought to induce genomic instability resulting in accumulation of mutations [95].

6.7 Mechanisms of Oncogenesis

Persistent viruses encode well-characterized oncogenes but are rarely directly oncogenic. Rather, life-long infection by these members of the human virome is typically only the first step of many that lead to carcinogenesis. Unraveling the role of these viruses in hematologic malignancies has been a challenge for many scientists over the last 50 years. Through that research, some common mechanisms have been elucidated.

The age at which the persistent viral infection is acquired impacts subsequent cancer risk. For example, while early age at infection with EBV is asymptomatic [8, 10, 101], this also increases the risk for endemic Burkitt's lymphoma [102]. In contrast, delayed infection with EBV until young adulthood leads to infectious mononucleosis, a self-limiting lymphoproliferative disease but it also is associated with an increased risk for Hodgkin's lymphoma [103, 104]. Similarly, infection with HTLV-1 through breastfeeding increases the risk for ATL [105, 106], while delay of infection to later in life results in increased risk of tropical spastic paraparesis.

Why the age of infection leads to differential risk for hematologic malignancy is not well understood. In regard to BL, one possible mechanism would be through the increased number of latently infected circulating B cells [10]. Although these cells are not malignant, the expanded pool of latently infected cells would drive a stochastic balance whereby the chance for a secondary oncogenic hit increases. *P. falciparum* induces an enzyme, activation induced deaminase (AID), that has been shown in mouse models to drive the c-myc translocation characteristic of BL [107]. AID is elevated in circulating B cells in children living in areas where malaria is endemic and in tonsils of children from malaria endemic regions [108, 109]. AID is also elevated in peripheral blood prior to emergence of NHL in HIV/AIDs patients [110] suggesting that sustained activation of this enzyme in B cells is a common risk factor for B-cell lymphomagenesis. High HTLV-1 viral loads are also seen as a risk factor for ATL [111].

In all of these viral infections, there is either continual virus production as is the case with HCV or reactivation of the virus from latency as with HTLV-1, EBV and KSHV. This can lead to chronic antigen exposure throughout the life of the host and

potentially driving exhaustion of the adaptive CD8+ T cell response to these pathogens [112–114]. The loss of the CD8+ T cell response is thought to result in failure to clear pre-malignant cells that then are driven to malignancy through expression of viral oncogenes. That many of these malignancies only occur long after the primary infection and patients with these lymphomas have exhausted viral specific T cells [115, 116] supports this model. In addition, studies in both EBV [117] and HTLV-1 [118] infected lymphocytes reveal transient expression of EBNA-1 or Tax, respectively suggesting an additional escape mechanism from CTL responses.

The above speaks to the immune cost for containing these members of our virome. With the loss of immunity due either to iatrogenic effects as a consequence of allogeneic stem cell and solid organ transplantation or due to HIV infection, the risk for emergence of lymphoproliferative diseases and lymphomas associated with these viruses is elevated [119–121]. Moreover, if immune function is not restored, the lymphoproliferative diseases can lead to lymphomas. This has been shown for patients with KSHV and MCD [51], HCV and mixed cryoglobulinemia [122], and EBV and LPD [123].

Many of the viral-associated hematologic malignancies require additional exogenous co-factors. For example, endemic Burkitt's lymphoma, a common pediatric cancer in sub-Saharan Africa, is etiologically linked to both EBV infection as well as *Plasmodium falciparum* malaria [126]. Primary effusion lymphoma is primarily found in patients that are co-infected with HIV and KSHV [50]. The EBV EBNA-1 protein was found to enhance HCV replication suggesting a potential interaction between these two members of the virome [124].

While the oncogenic capacity of these viruses is clear, how the viral encoded oncogenes contribute to the emergence of malignancy is a bit of a conundrum. This is because it is rarely possible in healthy infected individuals to detect the expression of the viral oncoproteins in infected cells. For example, while HTLV-1's Tax protein has oncogenic capacity, less than 5% of HTLV-1 infected cells isolated from peripheral blood express Tax and this can only be detected by sensitive PCR [125]. In EBV latently infected memory B cells—the site of long-term latency—only the EBNA-1 protein is detected and only then in memory B cells that have entered the cell cycle [117]. Moreover, it would be detrimental to long term persistence for these viruses to continuously express viral genes as the immune system would be able to eliminate those cells. One possible mechanism that would account for this paradox is the transient re-expression of viral oncoproteins that then can act as an initiator of oncogenesis by dysregulating key cellular pathways. During the transient activation, these viral oncoproteins could modulate cellular pathways including suppression of apoptosis and promotion of cell cycle.

A final thought—as we gain a better understanding of the role of microbiome in human health and disease, it seems likely that the microbiome will also have a role in leading to hematologic malignancy. The nature of that interaction is for future scientists to discover.

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Chapter 7
Association of Microbes with Breast Cancer

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Abstract Breast Cancer (BC) is one of the most prevalent of all cancers worldwide. It is a well-established disease with intrinsic and extrinsic risk factors. Over the past decades, scientists postulated a role for infectious agents and the resident microbiota as extrinsic risk factors for several types of cancers. Viruses may exert effects on the early stage of oncogenesis during the initiation or late stage through the regulation of cell proliferation and apoptosis. Bacteria within the host may interact with host cells, such as the epithelium and immune cells to affect development or progression of BC. For example, microbes may impact the host response, from somatic or immune cells, causing changes in inflammatory pathways and the tissue microenvironment, which may influence cancer development. Microbial communities composed of eukaryotic species, bacteria, fungi and viruses inhabit the human body and may contribute to cancer pathogenesis or prevention. This chapter describes studies related to the associations of BC and microorganisms present in humans discovered over the last decades.

Keywords Breast cancer · Breast microbiota · Microbiome · 16s sequencing · Bioinformatics

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7.1 Breast Cancer

Breast cancer (BC) is a heterogenous disease comprised of unique subtypes with distinct histological and molecular differences which dictate different therapies [1]. It is one of the most prevalent of all cancers worldwide [2]. In the United States, BC is recognized as the most prevalent noncutaneous cancer, and the sixth leading cause of death among all diseases/accidents [3].

7.2 Established Risk Factors

Many intrinsic and extrinsic risk factors have been well-established for BC. Intrinsic risk factors include early menarche, late menopause, parity (later age at first birth), positive family history of BC and individual high estrogen levels [4–6]. Five to ten percentage of BC cases (familial BC) are associated with the presence of variant mutant genes such as BRCA1 and BRCA2. Dietary habits such as fatty foods and alcohol consumption, as well as low levels of exercise, and use of the exogenous hormones are extrinsic factors that contribute to neoplastic transformation of mammary gland cells [7–11]. Over the past decades, scientists postulated a role for infectious agents and the resident microbiota of the host as extrinsic risk factors for several types of cancers [12, 13] including BC [12, 14–16]. Despite these studies, a clear role for microbes in BC remains unclear. A better understanding of an association of microbes with BC could contribute to the development of both primary and secondary prevention (early detection and management) measures.

7.3 Microbes and Breast Cancer

Over the past decades, the interest in infectious agents and the resident microbiota of the host has grown exponentially among investigators around the world [12, 13]. It is estimated that 15–20% of the worldwide cancer burden is due to viruses [17–19]. Furthermore, some pathogens have been implicated in promoting cancer by inflammatory injury, rather than by directly initiating carcinogenesis, such as *Helicobacter pylori*, associated with gastric cancer, Schistosoma helminthes with bladder cancer [20, 21] and *Fusobacterium nucleatum* in colorectal carcinoma [22, 23]. However, a role for microbes in BC remains unclear. Here, we review studies over the past decade regarding BC and microorganisms.

7.4 Viruses and Breast Cancer

Viral infection in human carcinogenesis has been well established [24]. Infection has been historically classified as carcinogenic since the beginning of the twentieth century when a virus was associated with sarcomas in chickens [25]. Since then,

science has discovered several viruses that exert effects on early stages of oncogenesis during initiation, or later stages involving cell proliferation and apoptosis. These findings provide several clinical implications [26]. Insights on the role of viruses in cancer provides new therapeutic targets for cancer and even precancerous states via vaccines and preventative measures [27]. The search for viruses in BC has been ongoing for decades. Here we describe studies highlighting specific viruses associated with BC. Some have been implicated to alter cell division, leading to uncontrolled proliferation and malignancy.

7.4.1 Human Endogenous Retrovirus Type K (HERV-K)

HERV-K are viral elements endogenous to the human genome. HERV-K comprise 30–50 members per haploid genome in humans and may contribute to the evolution of the human genome as well development human disease [28]. Two general types of HERV-K are known, distinguished by the absence (type 1) or the presence (type 2) of 292 nucleotides at the boundary of the putative *pol* and *env* genes [29]. The expression of HERV-K sequences are estimated to comprise 1–8% of the entire human genome [29, 30] and HERV-K is overexpressed in BC tissue at the mRNA and protein levels [31, 32]. Wang-Johanning et al. [33] showed type 1 and type 2 HERV-K *env* RNA was present in BC samples and not in normal breast tissues and cell lines, indicating the HERV-K gene loci may be transcriptionally activated in breast tumors [33, 34]. In fact, HERV-K- env expression was highly associated with poor prognosis in BC [33, 35]. However, the expression of HERV-K reverse transcriptase (HERV-K RT) was also reported in normal tissue adjacent to the breast tumor, suggesting either the possibility that HERV-K might be expressed very early in the tumorigenic process, although it is not necessarily an evidence of causation [35]. Studies showed that monoclonal antibodies [36] and chimeric antigen receptors [37] against HERV-K env protein were able to block growth and proliferation of human BC cells, leading to apoptosis and activation of the TP53 signaling pathway [36, 37]. Therefore, HERV-K envelope (env) protein may act as a tumor-associated antigen (TAA) for cancer vaccines, with antibodies possessing antitumor activity against cancer [36]. K-CAR T cells against HERV-K inhibit progression of BC as well as to reduce metastasis in mice compared to other treatments tested [38]. These findings in murine models suggest that targeting HERV-K may be therapeutic for BC highlighting its potential in promoting the disease.

7.4.2 Mouse Mammary Tumor-Like Virus (MMTV)/Human Mammary Tumor Virus (HMTV)

In 1936, Bittner and colleagues proposed that an unknown factor caused mammary tumors in adult mice [39]. Later, mouse mammary tumor virus (MMTV) was identified and a MMTV-like DNA sequence found in human BC among women (known as Human Mammary Tumor Virus/ HMTV) [40, 41]. Gene sequences with a

90–98% homology to mouse mammary tumor virus were found in BC tissue ranging from 0.8% (Vietnam) [42] to 36% (United States) and 74% (Tunisia) [43].

MMTV-like DNA sequences were found in breast tumor tissues in Australian, Mexican, American, Italian women [41, 42, 44–47] and in 62% of gestational (BC during pregnancy and/or lactation or within 1 year of delivery) [48] and inflammatory breast cancers [49]. In fact, Pogo and colleagues have been studying not only MMTV-like env but viral particles and proteins, and their findings implicate MMTV or HMTV in BC [50–52]. Moreover, in metastatic BC, viral DNA sequences of a β-retrovirus similar to MMTV were found in cells isolated from ascites and pleural effusions [52]. Recently, HMTV-env sequences were confirmed by PCR in Australian benign breast biopsies specimens from women prior the BC diagnosis taken 1–11 years earlier, evidencing MMTV-like virus as a possible causal role for the development of BC [53].

On the other hand, others report no association of MMTV-like virus in BC [54–58]. Morales-Sánchez found no evidence of HMTV in 86 samples of BC from Mexican women [56]. Similarly, none were detected in Japanese [55], Australian [57] and Austrian [58] cases of BC. Hence, the role of HMTV in BC remains uncertain.

7.4.3 Bovine Leukemia Virus (BLV)

Bovine leukemia virus (BLV) has been broadly investigated in agriculture [59–61]. BLV is an infectious virus known to spread through herds of cattle causing B-cell leukemia [62]. Previous findings demonstrated the presence of BLV antibodies in humans [63]. In 2014, the same group investigated the presence of BLV due the abundance of BLV DNA and proteins in mammary epithelium in cattle, and they found the BLV DNA in breast tissue without regard to diagnosis [64]. The next step was to evaluate tumor breast tissue, Buehring and colleagues performed a case-controlled study of 239 formalin-fixed paraffin embedded (FFPE) breast tissues with 114 diagnosed with BC. They reported the presence of BLV DNA in mammary epithelium with BC in 59% against 29% in normal controls, suggesting an association with BC [65]. Recently, Buehring's group studied 96 Australian women, which 50 patients had a history of BC, and they found 80% (40/50) of BLV DNA detected in the tissue, whereas 41% (19/46) of no history of BC the detection was also confirmed, These results corroborate with the previous findings in American women [66].

BLV is present in dairy and beef cattle as well as blood cells [67], yet it is not clear how transmission of BLV to humans occurs, since the pasteurization process eliminates the virus from cow's milk [68]. One might envision transmission by human ingestion of unpasteurized milk or raw beef from infected cows, or longitudinal transmission from an infected mother to her baby [69]. Additional studies are necessary to confirm the association of BLV with BC.

7.4.4 Human Papilloma Virus (HPV)

Papillomaviruses are DNA viruses that infect keratinocytes in stratified squamous and mucosal epithelia. Low risk types result in skin or mucosal lesions such as cutaneous warts and condylomas while high risk types are oncogenic [70]. The most prevalent high-risk types are HPV-16 and HPV-18, which account for 70% of cervical cancer case, and it has been strongly associated with oropharyngeal cancers [71]. In fact, the number of HPV-associated cases of head and neck cancers among nonsmoking middle-aged white females is increasing in the United States [72].

The HPV oncogenes E6 and E7 are encoded by most papillomaviruses and enable viral synthesis and replication [73]. E6–7 regions of human papillomavirus types 16 and 18 express oncoproteins that will interact with cellular proteins, regulate the cell cycle and/or interfere with the host cell DNA [74, 75]. The viral oncoproteins E6 and E7, which inactivate tumor suppressor genes TP53 and RB, are known for being expressed in cervical cancer [73, 76, 77].

Regarding BC, an association with HPV is unclear. Some evidence for HPV in BC has been reported [78–81]. For instance, a study performed using 54 fresh frozen BC found 50% of the samples with presence of HPV [78]. Through in situ PCR, a technique that combines the sensitivity of PCR or RT-amplification along with the ability to perform morphological analysis associated with standard PCR, HPV was found in the nuclei of eight of 28 BC specimens while three of 17 normal breast specimens were HPV positive [79]. In Argentina, 61 fresh frozen BC specimens were analyzed by PCR and the results show 28% (16/61) positivity for HPV DNA, suggesting that HPV may have a biological role in BC [81]. HPV18 was present in the majority of positive BC cases [78, 79].

Despite reports of the presence of HPV in BC, investigators applying other methods did not detect HPV [82–85]. A large cohort of 228 breast tumors and 142 blood was used through different PCR methods and was observed in Indian women lack of detection for HPV DNA either in tumor or in the blood [84]. A study using Next Generation Sequencing failed in finding expression of HPV transcripts in 80 BC samples, although 16% of breast tumors confirmed the presence of DNA. Therefore the viral genomes are present but are not transcribed, hence, functionally inactive [83]. Furthermore, it is possible that the level of HPV in breast tumors is so low that it may not be oncogenic (a mean of 5.4 copies per 10^4 cells) [83, 86, 87]. On the other hand, even such a low HPV load might be pathogenic if stimulated by other factors such as other viruses or molecular mechanisms to enhance the oncogenic potential of HPV [88–90].

Among these molecular mechanisms are exogenous and endogenous mutagen exposures causing altered DNA sequences. For example, the APOBEC gene family [91, 92], including the APOBEC3 genes (A,B,C,D,F,G,H) and APOBEC4 genes, encodes proteins with conserved DNA cytosine deaminase domains [92]. The overexpression of cytosine deaminases can lead to mutations responsible for the transformation of the cells [93, 94]. While HPV can induce overexpression of APOBEC3B gene leading to a more aggressive phenotype in infected breast epithelial cells [95],

Tsuboi and colleagues reported an association between APOBEC3B expression and BC that was not related to HPV infection [96]. Finally, other factors such as sexual behavior or different geographic regions may contribute to whether HPV is present in BC tissue, and thus perhaps the role for HPV in BC may differ based on other patient characteristics [84, 87, 97].

7.4.5 Epstein Barr Virus (EBV)

Epstein Barr Virus (EBV) was isolated and characterized as a herpes group virus from lymphoblastoid cells of the B lineage in African Burkitt's lymphoma, non-Hodgkin and Hodgkin lymphomas and nasopharyngeal carcinoma [98, 99]. It is estimated that more than 90% of the adult population shows serological evidence of past infection with EBV [99]. The most known EBVs are types A (aka 1 or B95-8 strain) and B (aka 2 or AG876 strain), which have been distinguished based upon genetic signature in the Epstein Barr nuclear antigens (EBNAs) sequence [100]. Its carcinogenic potential was demonstrated by Kempkes and colleagues in 1995 when the researchers were able to immortalize B cells in vitro due to EBV infection [101]. Cancer cells infected with EBV promote rapid growth in initial stages but ultimately EBV infection did not impact the final tumor size [102]. EBV may also induce overexpression of the APOBEC family genes predisposing mammary epithelial cells to malignant transformation [103].

In 1995, Labrecque and colleagues first reported the presence of EBV in BC tissue [104]. Since then, authors have been attempting to identify EBV in BC with data still unclear and matter of debate for several years [105–107]. Glenn and colleagues reported the presence of EBV sequences in 68% of fresh frozen invasive BC (from 50 unselected invasive BC) using in situ PCR technique [86]. Later, a study with 117 BC specimens in France demonstrated that although EBV was associated with the most aggressive BC phenotypes (38/32.5% positivity), it did not exert influence on the disease prognosis [108]. A study with 85 breast tumor biopsies over an 87-month follow-up period, showed 25.8% positivity for EBV DNA with the replicative form correlating with poor outcomes, whereas the latent form conferred a better survival outcome in BC patients, possibly through activation of non-specific anti-tumoral immune responses [109]. Recently, Glaser and colleagues using several q-PCR assays, found 38 (out of 127 specimens) EBV-positive breast tumors associated with poorer survival in older women with BC [110]. In an Egyptian cohort, no statistically significant association of EBV with BC was found, but EBV presence was correlated with tumor aggressiveness [111].

On the other hand, Perrigoue and colleagues revealed that EBV occurs within <1% of the cells suggesting that the presence of EBV was not more common in tumor cells than the paired normal cells [112]. This is in accordance with Thorne and colleagues who found low levels EBV DNA in the invasive BC FFPE specimens, not exceeding 11 copies per 100,000 cells, suggesting that only a few cells were infected [113]. The variability of techniques used for viral detection, as well as

of the patients studied leads to challenges in identifying the virus [114]. Therefore, the presence of EBV and its role in overall BC survival requires more study.

7.4.6 Human Cytomegalovirus (HCMV)

Human Cytomegalovirus (HCMV) or human herpes virus 5, is a member of *herpesviridae* family responsible for infecting 50–90% of general population worldwide with acute, persistent or latent infections [115]. The presence of HCMV in tumors has been attested [116–118]; in 1997 Richardson and colleagues hypothesized that late exposure to HCMV results in higher risk of developing BC [119]. Later, they tested plasma from BC (N = 208) and control subjects (N = 169) from a population-based case-control study of Australian women under 40 years old and found an association between HCMV IgG levels and BC in young women, further implicating HCMV [120]. El-Shinawi and colleagues studied biopsies and blood samples for HCMV infection in 28 inflammatory BC patients (IBC) and 49 non-IBC. The results revealed the IgG serum titer was higher in IBC when compared to non-IBC patients. El-Shinawi also reported HCMV DNA in BC tissues and suggested a role for HCMV in IBC by activation of transcription factor NF-kB signaling [121], known for its association with poor prognosis [122, 123]. Others showed strong evidence of HCMV in primary BC samples [124, 125] with expression of viral protein in sentinel lymph node of BC metastases [125].

Recently, studies demonstrated a significant role for cmvIL10 (a viral cytokine that binds to the IL-10 receptor) to activate immune suppressive functions, promoting malignancy and uncontrolled proliferation of BC cells line resistant to apoptosis [126–128]. These results suggest the potential for cmvIL-10 in enhancing the aggressiveness in BC phenotypes and support the inclusion of an antiviral treatment as an adjuvant therapy to BC patients.

On the other hand, a study evaluated 27 specimens of FFPE breast carcinomas (stages II, III, IV), where only 7.4% showed positivity for HCMV [129]. Despite the association of BC with serological evidence HCMV past infection [120], Richardson and colleagues later were not able to detect HCMV in 70/70 frozen BC tissues and only 2/70 (3%) positive for the paired normal breast tissue [130]. Lastly, Antonsson and colleagues were unable to detect HCMV in 54/54 frozen BC tissues [131].

7.4.7 Polyomaviruses

Polyoma (Py) are small nuclear DNA viruses that infect a diverse range of body sites, particularly epithelial tissues [132, 133]. The number of human Polyomaviruses has expanded over the last 5 years, with ten new viruses recently described [132, 134]. Polyomaviruses have epithelial tropism and are frequently associated with circulating leukocytes, which suggests a plausible route to breast tissue [135, 136].

Polyomaviruses can cause tumors in animal models, and immortalize animal and human cells in culture including mammary glands [134, 137] leading to genetic alterations [138]. The mechanism of Polyomavirus-induced oncogenesis is attributed to the expression of oncogenic proteins which disrupt the cell cycle [134, 139].

Hachana and colleagues found positivity in 28 of 112 invasive ductal carcinomas for one type of Polyomavirus using PCR analysis [140]. Py viruses were present in the tumor microenvironment but not the paired healthy tissue, suggesting a role in BC [140]. In contrast, Antosson and colleagues evaluated the prevalence of polyomaviruses and Herpes virus in 54 BC fresh frozen tissues. The results showed no detection for polyomaviruses in the invasive ductal carcinomas [131]. Another study using 155 paraffin-embedded malignant tumors from Algeria, found only one positive tumor (0.65%) for Polyomavirus [141]. Further investigations on the association of Py virus with BC may be warranted given its well established role in other human epithelial cancers [142].

Given the inability to detect different viruses in tumor tissue, it is possible that the damage by viruses was incurred at a much earlier state, referred to as the "hit and run" hypothesis [143, 144]. The virus is able to infect the cell causing a genetic instability and or epigenetic dysregulation through an initial "hit" while maintenance of the transformed state is compatible with the loss or "run" of viral genome [143–145]. Therefore "viral negative" tumors could have been induced by viruses [129, 143, 146], in that viruses can initiate mutations, yet they may not be required for the maintenance of cell transformation in a susceptible microenvironment.

Despite our established knowledge of risk factors such as age, post menopause, geographic locations, family history for BC and efforts by scientists worldwide to identify causes of BC, the cause of most BC cases remains unclear [147]. With this in mind, the role of virus infection in BC has been challenging the scientific community for decades [14]. The studies still lack consensus [114], however, technology and modern techniques should improve our ability to determine the role of viruses in BC, their potential to interact with the human genome or in concert with other viruses. For example, co-prevalence of EBV and high–risk of HPV was detected in 52% of breast tumor samples (N = 108) with invasive phenotype [148] suggesting a potential role for collaboration between viruses to contribute to breast carcinogenesis.

A role for viruses in BC and the potential for exploiting viruses as therapeutic targets for prevention or clinical intervention in BC remains to be determined.

7.5 Bacteria and Breast Cancer

Sanger and Coulson, along with Maxam and Gilbert were the pioneers to sequence DNA by chain termination and fragmentation, respectively [149, 150]. Sanger sequencing was popularly accepted the past 30 years since the chemicals and radioisotopes for the experiment were less toxic and complex when compared to Maxam's and Gilbert's technique [151]. The development of Next Generation Sequencing

(NGS) technology [152] allows analyses down to a complex microbial genomic level independent of any need for *in* vitro cultures [153]. With the advent of NGS, our ability to comprehensively describe the human microbiota of various organ systems or tissues has provided an explosion of associations between some bacterial species with human cancers [12, 153]. Recent NGS studies of BC patient specimens suggest some bacteria might be associated with BC.

7.5.1 Gut Microbiota and Breast Cancer

As early as the 1970s, Hill and colleagues hypothesized that the contents of the gastrointestinal tract and the microbiota who metabolize them could be involved in BC through the consumption of a diet rich in fatty foods responsible for high levels of estrogens derived from the biliary steroids present in the colon [154]. Studies showed that gut microbes are able to modulate and increase systemic estrogen levels through β-glucuronidase activity, involved in several chemical reactions resulting in reabsorption of free estrogen into blood stream through the portal vein [155–157]. High blood levels of estrogen are strongly associated with higher risk for BC in postmenopausal women [158] and such levels of systemic estrogens are linked to gut dysbiosis [159].

Notably, the functions of the microbiota may be unrelated to systemic estrogen levels, implying another role of gut microbes independent of estrogen-pathways related to BC development [160]. Goedert and colleagues investigated the gut microbiota in 48 pretreatment postmenopausal BC, and 48 healthy patients. The fecal microbiota of case patients compared to control showed less diversity and compositionally different when compared to healthy controls. Therefore, BC patients may have an altered gut microbiota composition and estrogen-independent low diversity in their microbiota [160].

Bard and colleagues also observed differential composition of the gut microbes in BC. Thirty-two patients with early stage of BC before any therapeutic intervention showed a difference in composition of gut microbiome according to clinical features and BMI [161]. Later, the same group performed a study with 31 patients with BC and they found Blautia sp. associated with the most severe clinical stage and prognostic grade [162].

In addition to this complex and dynamic contribution from gut microbes is the potential for microbes to influence the host immune system [163, 164]. This interplay has evolved to promote the ability for bacteria to coexist in the gastrointestinal lumen, leading to a sort of immune tolerance of the host immune system and the host gut microbiota [165]. Such mutualism creates an interaction responsible for modulating the inflammatory processes and defense mechanisms [165, 166], implicating gut microbes to affect antitumor immunity.

In fact, gut microbiota may influence immune cells contributing to the development of BC [163, 167]. For example, induction of mammary tumors with *Helicobacter hepaticus* by gastric gavage in predisposed mice was inhibited by

depletion of neutrophils, suggesting cooperation between innate immune responses and gut microbiota for BC development [163]. Likewise, certain gut microbiota may influence the ability of immune cells to protect against BC. *Lactobacillus reuteri* added into drinking water inhibited carcinogenesis and boosted CD25+ Foxp3-Tregs in mammary cancer-susceptible mice on a high fat diet [168]. Further clinical studies are necessary to determine how the gut microbiota might interfere in BC development. Taken together, the association of gut microbes with BC and tumor immunity warrants more study.

7.5.2 Antibiotics Associated with Breast Cancer

7.5.2.1 Antibiotics

If the gut microbes may contribute to BC in a protective or carcinogenic way, it is likely that the alteration of gut microbes with antibiotics should affect BC pathogenesis. However, the association of BC with antibiotic use is unclear. Several studies using large population databases have conflicting results (Table 7.1). A study in Finland published in 1999 reported 1.74 higher BC risk (95% CI 1.13–2.68) associated with antibiotic use for urinary tract infections in women under 50 years of age [169]. Although the study had some limitations such as antibiotic for UTI use only without description of other antibiotics, or not taking in consideration other risk factors such as previous benign breast disease, hormone replacement or family history, the study suggested an unusual risk factor distant from the tumor site with implications on the BC.

Following this study, others attempted to replicate the finding in similar studies of different populations. Velicer and colleagues evaluated more than 10,000 women, and they found that increasing cumulative days and antibiotic prescriptions were associated with increased risk of BC (2.07, 95% CI 1.48–2.89) [170]. Didham and colleagues studied 6678 cancer patients in New Zealand where 700 were diagnosed with BC. The study showed that penicillin use was associated with 1.07 (95% CI 1.02–1.13) times higher incidence of BC [171]. Friedman and colleagues evaluated data from 2.1 million women in 9.4 years of follow-up, and they observed a slightly increased risk (1,14, 95% CI 1.10–1.18) of BC associated with tetracycline and macrolide use [174]. In Canada, investigators evaluated more than 3000 BC cases and they observed a dose-dependent increase in BC risk in association with antibiotic exposure up to 15 years, although the lack of timing and class effects on this association suggest a non-causal relationship [176]. In 2013, Wirtz conducted a retrospective cohort study with 4216 women ≥18 years old with incident stage I/II BC for 6.7 years. They showed a modest risk of secondary BC events (HR 1.15, 95% CI 0.88–1.50) among frequent antibiotic users when compared to nonusers, but the association was not significant [177]. A similar finding was also described by Boursi and colleagues in a nested case-control study where BC was modestly associated with exposure to sulphonamides [adjusted OR of 1.2 (95% CI 1.0–1.4)] [178].

Table 7.1 Summary of studies with antibiotics associated with Breast Cancer

	Type of study	Number of subjects	Association with BC	Conclusion
1. Knekt et al. [169]	Survey cohort	9461 women—18-year follow-up period	RR for women under 50 years old: 1.47 (95% CI 0.73–2.97) Older women: 0.97 (95% CI 0.59–1.58)	Premenopausal women using long-term medication for UTI show a possible elevated risk of BC
2. Velicer et al. [170]	Case control study	2266 older than 19 years old (primary, invasive BC), 7953 randomly female health plan members	Association between extent of antibiotic use and risk of BC	Use of antibiotics was associated with increased risk of incident and fatal BC
3. Didham et al. [171]	Nested case-control study.	6678 patients identified with cancer	Slightly increased odds ratio OR (95% CI) for BC was seen with penicillin, 1.07	Antibiotic exposure represents a confounding factor rather than a causation for BC
4. Sorensen et al. [172]	Case-control study	2728 BC cases and 27,280 controls	BC with more than 10 prescriptions for antibiotics 1.0 (95% CI 0.86–1.15)	Use of antibiotic was not associated with increased risk of BC
5. Kaye and Jick [173]	Case-control study	1268 cases of BC and 6291 controls	0, 1–50, 51–100, 101–500 and 501 or more accumulative days 1.0 ref., 1.0 (0.9–1.2), 0.9 (0.7–1.3), and 1.2 (0.6–2.4).	Antibiotic use was not associated with an increased risk of breast cancer.
6. Garcia Rodrigues and Gonzales-Perez [179]	Nested case-control study	3708 BC cases and 20,000 controls	For categories of increasing cumulative days of use (1–50, 51–100, 101–500, and > or = 501 days), the corresponding odds ratios were 1.0 (95% CI: 0.9–1.1), 1.0 (0.8–1.1), 0.9 (0.7–1.0), and 1.2 (0.9–1.6) (p = 0.31 for trend)	Antibiotic use was not associated with an increased risk of breast cancer

(continued)

Table 7.1 (continued)

	Type of study	Number of subjects	Association with BC	Conclusion
7. Friedman et al. [174]	Cox proportional hazards analysis	2,130,829 women subscribers of a health care program from outpatient pharmacies for 9.4 years of follow up	18.521 developed BC Any antibiotic: 95% CI 1.12 (1.10–1.18). Tetracyclines and macrolides: HR, 1.23 (1.11–1.36) and 1.16 (0.98–1.36)	Most antibiotic use was associated with little increase of BC in up to 9 years of follow-up
8. Velicer et al. [175]	Case-control study	2266 women with primary, invasive BC	Compared to non-use, antibiotic use prior to breast cancer diagnosis was not associated with BC	Antibiotic use prior to BC diagnosis was not statistically significantly associated with tumor features
9. Tamim et al. [176]	Nested case-control study	3099 BC and 12,396 controls	The incidence of BC was higher in subjects who had more antibiotic prescriptions during the 1–15 years prior the index date (RRs = 1/50, 1.63, 1.71 and 1.79 for the four quartiles p-trend = 0.0001	Dose-dependent increase in BC risk was associated with the antibiotic exposure up to 15 years
10. Wirtz et al. [177]	Retrospective cohort study	4216 women for a median of 6.7 years with secondary breast cancer events (SBCE)	SBCE CI 95% 1.15, (0.88–1.50) among frequent antibiotic users compared to nonusers	Frequent antibiotic use may be associated with modestly elevated risk of SBCE but the association was not significant
11. Boursi et al. [178]	Nested case-control study	31,252 BC cases and 123,285 controls	BC associated with exposure to Sulphonamides 95% CI 1.2 (1.0–1.4)	A modest risk exposure to Sulphonamides was associated to BC

In contrast, Velicer's group later reported no association of antibiotic use with BC characteristics such as tumor grade, stage, size, histology and estrogen receptor status in 2266 women in US [175] along with other case-control studies. Another nested case-control control analysis included 734,899 women with 3708 women diagnosed with BC and 20,000 frequency matched controls did not show association with BC risk [179]. In addition, the use of antibiotics among 2728 women with previous history of BC and 27,280 women with no history of antibiotic use did not show an increased risk associated with use of antibiotics compared to nonuse [172]. Lastly, Kaye and Jick identified 1268 cases of BC and 6291 controls from the U.K. General practice research database, and they also did not support the hypothesis that antibiotic use is associated with BC risk [179].

While the reports are conflicting, antibiotic administration can result in gut microbiota dysbiosis, leading to a disturbance in bacterial composition. Broad-spectrum antibiotics can affect the abundance of 30% of the bacteria in the gut community [180], therefore, antibiotic exposure alters the physiological balance and influences the regulation of immunity and metabolism [181]. In fact, intestinal microbiota is critical in vaccine effectiveness due the activation of the immune system through pattern recognition receptors [182]. Consequently, over the course of cancer treatment, antibiotics are administered frequently and may interfere with the response to chemotherapy or immunotherapy [181].

A large spectrum of studies show the importance of host microbes in cancer is pertinent, but more work is required to determine the mechanisms by which antibiotics and specific microbes might impact BC pathogenesis or course of disease.

7.5.3 Breast Microbiota and Breast Cancer

In contrast to viruses, studies of the presence of bacteria in breast tissue and how they interact with the normal and tumor microenvironment have just begun. Xuan and colleagues were the first to report the breast microbiome from the study of 16S V4 amplicon paired-end reads from post-menopausal ER-positive BC patients using NGS in 20 paired FFPE tumors and adjacent normal breast tissue. The breast microbiome showed predominance of Proteobacteria, followed by Firmicutes, Actinobacteria and then Bacteroidetes in both tumors and healthy-adjacent breast tissue [16]. Interestingly they found differences between BC and paired adjacent normal tissues, mainly due to bacteria in the Sphingomonadaceae family. A relatively higher abundance of *Sphingomonas yanoikuyae* was observed in adjacent normal breast tissue compared to BC tissue, while *Methylobacterium radiotolerans* was the highest found at the site of tumor. The association of higher bacterial DNA levels with lower staged BC suggests a potential protective role, perhaps through stimulation of host immunosurveillance and antitumor responses. However this association does not indicate causality and further studies are warranted to investigate the pathophysiologic relevance of the local host/microbe interaction in breast cancer.

Since that first report, several others have reported the presence of bacterial DNA in breast tissue by either array-hybridization technology or DNA sequencing. A bacterial signature was found in patients with triple negative BC, with members from Arcanobacterium, Brevundimonas and Sphingobacteria had the highest detection rate in 100 FFPE specimens of triple negative BC (PathoChip) [183]. Thompson and colleagues mined TCGA RNAseq data to perform meta-transcriptomics from six ER+ breast cancer tissues [184]. Since TCGA data was generated without the initial intention of microbiome studies, results should be validated. The group also performed 16S sequencing and also identified *Sphingomonas* genus, consistent with other studies [16, 183, 185].

A recent study of 16S V3-4 amplicon paired-end reads was performed using fresh frozen tissue from a combination of 17 breast tumors and 22 normal healthy fresh frozen tissues from invasive BC patients. These samples showed a lower abundance of *Methylobacterium* compared to 24 breast tissues from non-cancer patients undergoing bilateral reduction mammoplasty or mastopexy [186]. Although comparative levels of *Methylobacterium* may differ in various disease states (cancer vs healthy) it is interesting to note that the same genus was identified in two independent studies of breast tissue [16, 186]. Again, further studies are warranted to investigate the roles of specific bacterial in breast carcinogenesis.

A handful of studies have described the microbiome of normal breast tissue. In line with Xuan et al., Urbaniak and colleagues reported a high abundance of Proteobacteria followed by Firmicutes phyla in normal and BC adjacent tissue of 81 women from Canada and Ireland using 16S V6 sequencing with Ion Torrent technology [187], unlike all others which used Illumina sequencing by synthesis (SBS) technology [15, 16, 183–186, 188]. At the genus level, bacteria identified in healthy breast tissue from these women showed an abundance of *Bacillus* (11.4%), *Acinetobacter* (10.0%) and unclassified *Enterobacteriaceae* (8.3%) in Canadian women while 30.8% of Enterobacteriaceae, 12.7% *Staphylococcus* and 12.1% of *Listeria welshimeri* were found in Irish women. Although the study did not sequence tumor tissue, a higher abundance of *Escherichia coli* was detected in healthy adjacent tissue (taken 5 cm from tumor) from women with cancer when compared to healthy controls [187].

Urbaniak also reported studies of fresh normal adjacent breast tissue from women with BC, benign tumors, and healthy patients (cosmetic breast reductions or enhancements) by 16S V6 Illumina MiSeq [15]. Normal adjacent tissue from BC women showed higher presence of genus *Bacillus*, *Enterobacteriaceae* and *Staphylococcus* with more similarities with benign tumor tissue than when compared to healthy controls. The authors propose that *Escherichia coli* (Proteobacteria phylum) and *Staphylococcus epidermidis* (Firmicutes phylum) isolated from the normal adjacent healthy tissue from BC patients could play a role in carcinogenesis [15]. Regarding the healthy controls, Lactic Acid Bacteria (LAB) used in food fermentation (yogurt and cheese), genus *Lactococcus* and *Streptococcus* were found in abundance [189] which may be protective through stimulation of immune cells controlling tumor growth [190–192]. Hieken and colleagues performed an investigation of 16S V3-5 paired end amplicon sequencing of normal healthy adjacent snap frozen tissue from 13 benign breast disease and 15 BC patients. The study reported differences comparing healthy tissue of benign disease and BC only with unweighted UniFrac distance ($p = 0.0009$), while the weighted UniFrac distance was not significantly different. While this result is intriguing, the statistically different *unweighted* UniFrac distance could be a technical artifact from oversampling data at a high sequencing depth resulting in the assignment and analysis of spuriously identified unique microbes, or operational taxonomic units, OTUs (see Sect. 7.5.4). Interestingly, the microbiome from breast tissue was statistically different from the overlying breast skin, consistent with their distinct environments and ecosystems [188]. Therefore, several questions remain regarding the intratumoral

microbiome of BC, given the intriguing findings of normal healthy adjacent tissues of BC patients.

Lastly, studies investigating the existence of microbes in the breast ductal system sampled with nipple aspirate fluid (NAF) found significant differences in bacterial composition by 16S V4 paired end sequencing between healthy controls compared to women who had previously had BC. *Alistipes genus* was enriched in BC NAF samples whereas *Sphingomonas yanoikuyae* was enriched in healthy samples. As expected, the nipple skin samples from the same study showed no differences in bacterial diversity between healthy women and BC patients, suggesting that the nipple skin microbiome has no association with BC [185].

7.5.4 Important Considerations with Interpretation of Various Microbiome Studies

The presence of a bacterial microbiome has clearly been shown to be present in the breast [15, 16, 183–188]. However, the exact identities of relevant microbes associated with health and disease remain to be determined. It is highly likely that the identities will be less relevant than particular genes, molecules or macromolecules, and pathways present as a result of the microbes' presence.

NGS technology has opened this new concept and field in breast cancer, the *presence* of bacteria in the breast. However, all initial findings require further study and this method comes with many weaknesses. First, the breast tissue microbiome is one of very low bacterial biomass, which presents a challenge that even the slightest level of contaminant can dramatically affect the results and ability to compare different studies to each other. Different studies use different collection methods, different DNA extraction kits, and also have different levels of sterility (Table 7.2). In fact the DNA extraction kits may even have their own contaminants [193]: their own "kitome" or contaminants may be introduced from other aspects of DNA extraction. It is critical to consider any sequences present in samples which may reflect the "kitome" amplified in the samples which were run with no tissue included (no template controls, or NTCs).

Some studies test for the presence of bacterial DNA after amplification by running an agarose gel prior to sequencing. When a band is absent, it may be concluded that there were no contaminants in these NTCs and these samples may then be excluded from further testing and no sequencing is performed on these NTC negative controls. However, sequencing is relatively much more sensitive than the ability to visualize a nucleic acid band on a gel; the omission of these negative controls from the sequencing reactions simply can lead to the omission of relevant "baseline" microbes due to contaminants which would then be included in analyses. On the other hand, the most stringent subtractions may lead to loss of relevant data. For example, Urbaniak and colleagues clustered their samples based on sequence similarity and removed approximately a third of the breast tissue samples that grouped

Table 7.2 Summary of breast microbiome studies

Sample source	Sample type	Variable region	Platform	DNA extraction kit	Reference database	Data reference	Reference
FFPE	– Cancer tissue (postmenopausal, ER+; multifocal, multicentric BC) – Normal adjacent paired from same patient	V4	Illumina MiSeq	QIAamp DNA FFPE Tissue kit	GreenGenes	PRJEB4755	[16]
FFPE	– Cancer tissue (TNBC) – Normal adjacent – Healthy	N/A	Agilent Pathochip	Nextera XT sample preparation kit	—	Unavailable	[183]
Fresh frozen	– Normal adjacent to cancerous tumor – Normal adjacent to benign tumor – Healthy	V6	Ion Torrent	Gene-Elute mammalian genomic DNA miniprep kit (Sigma-Aldrich)	RDP/ GreenGenes	Unavailable	[187]
Fresh frozen	– Normal adjacent – Benign non-atypia	V3–V5	Illumina MiSeq	PowerSoil DNA Isolation Kit (MoBio Laboratories Inc. Cat. 12,888)	GreenGenes (v13.5)	SRP080294	[188]
Fresh frozen	– Normal adjacent – Healthy	V6	Illumina MiSeq	QIAamp DNA stool kit; Qiagen	SILVA	SRP076038	[15]
Frozen sections	– Cancer tissue ER+	V3–V5	Illumina	The MoBio PowerSoil® DNA Isolation Kit	GreenGenes 12.10	Unavailable	[184]
Flash frozen	– Cancer tissue – Normal adjacent – Healthy	V3–V4	Illumina MiSeq	PowerMag Microbiome RNA/ DNA Isolation Kit	GreenGenes	Unavailable	[186]
Nipple aspirate fluid	– History of breast cancer – Healthy	V4	Illumina MiSeq	QIAamp DNA Mini Kit (Qiagen)	SILVA v119	SRP071608	[185]

with the environmental controls [15]. Chan and colleagues removed every OTU that was found in their no template controls [185]. These methods of dealing with potential contaminants may have been too stringent, potentially losing relevant reads.

Furthermore, the way the OTUs are identified and assigned taxonomy may differ with the choice of variable regions of the 16S ribosome gene selected for amplicon sequencing. While each choice of variable region(s) to sequence have their own strengths and weaknesses (beyond the scope of this review), they have different lengths with different thresholds for quality filtering and clustering (grouping) of OTUs. This is further complicated by the differences in reference databases (GreenGenes versus SILVA) and whether or not the OTUs were defined by closed-reference, open-reference, or de-novo clustering. As such, the number of different OTUs and the magnitude of the alpha diversity indices are not directly comparable across studies.

There is also the issue of batch effects. These may be observed within experiments showing differences between sequencing lanes. Similarly biological and other confounders associated with collection may also attribute to differences in microbiome results. Samples may also have technical batch effects from different DNA extraction dates. These should be considered in the OTU-level analysis as well as for alpha diversity and beta diversity analyses that assess the bacterial community composition. For example, one study's most prominent finding was a geographical difference between Canadian and Irish breast tissue microbiome [187]; however, this may be due to different laboratories, or simply different hospitals. Hieken and colleagues, studied BBD and DCIS patients who were significantly different by age and menopausal status [188]. Lastly, Wang and colleagues compared specimens from healthy and cancer patients with statistical differences in age, menopausal status, as well as race and BMI [186]. The lack of stratification of these other clinical variables may lead to spurious associations with disease and should be considered in the experimental design of future studies.

Lastly, it is important to consider the number of reads sampled (rarefaction depth). In studies with unusually low numbers of reads sampled, the proportions (or relative abundances) of each identified microbe, or operational taxonomic unit (OTU), will have high variation based on random sampling chance. For example, in a study which analyzed samples rarefied to a *relatively low* 60 reads, (i.e. 60 reads were randomly sampled prior to calculating the proportion of OTUs per sample) [186], it is possible that another subsequent and independent analysis of the exact same raw sequencing files could lead to completely different results after randomly sampling another 60 reads. *Oversampling* the number of sequencing reads can also lead to unreliable conclusions, as more OTUs are generated from sequencing errors. This well-known phenomenon is observed when sequencing longer fragments since the DNA polymerase sequencing errors increase with longer read lengths and there is less of an overlap between the paired-ends to verify the bases. Therefore, the number of OTUs per sample may steadily increase with the number of reads sampled. A robust analysis to prove reproducibility of results should be performed to demonstrate that any given finding is consistent regardless of low, medium, or high sampling depths.

7.6 Summary

A role for microbes and cancer in humans has been proven in some specific cases and types of cancer, but not yet for breast cancer. Despite decades of searching, with a huge effort to identify association of various viruses with BC, a clear role for viruses has not been determined. Similarly, the advent of NGS to sample in the breast tissue or ductal system in an unbiased manner has led to a new field describing the bacterial breast microbiome, however more work is required to better understand the role of bacteria in the local breast microenvironment and BC development and/or prevention. In addition, the role for bacteria in distant sites such as the gastrointestinal tract and their ability to influence the systemic immune response, impacting antitumor immunity also remains to be determined. A better understanding of an association of microbes with BC could contribute to the development of both primary and secondary prevention (early detection and management) measures, and therefore further studies are necessary.

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Chapter 8
The Microbiome Associated with Lung Cancer

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Abstract Recent studies on the lung microbiome have renewed the interest in understanding the relationship between microbes and lung diseases. The complex symbiotic relationship between microbiota and host have led researchers to postulate that many host diseases, including cancer, are directly associated with the commensal microbiome. Evidence suggests that the lung microbiome may contribute to local host inflammatory changes, which include the Th17 response. In lung cancer, studies suggest that lung dysbiosis may affect different stages of carcinogenesis. In this article, we review the latest knowledge gained from microbiome studies and explore possible mechanisms of microbe-host interaction that may have relevance to lung cancer pathogenesis.

Keywords Lung · Microbiome · Lung cancer · Inflammation

Currently, there is mounting evidence that supports a potential role for microbes in malignant transformation of cells in mucosae. Fusobacterium nucleatum is enriched in the gut of colorectal cancer patients [1] (Numerous studies have shown a direct relationship between Human Papilloma Virus and the incidence of cervical cancer [2] Helicobacter pylori has been accepted to be an important cause of gastric cancer and gastric MALT lymphomas [3] In addition, lung cancer is currently associated with diseases in which chronic colonization of the airways is common, such as in chronic obstructive pulmonary disease (COPD) [4] and HIV [5].

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8.1 Lung Microbiome: The Fall of the Sterility Dogma and Its Multiple Associations with Disease States

Since the discovery of bacteria, it has been appreciated that the vast majority of the human mucosae are inhabited by a complex set of microbes (collectively called microbiome) that affect our healthy homeostasis as well as different diseased states. The lower airways, however, have long been thought to be sterile, despite being the mucosae with the largest surface area exposed to the air and in anatomical continuity with another mucosa with one of the largest bacterial burden in our bodies: the oral cavity. However, several years ago, it was recognized that microaspiration is a common event in health [6] and it is increased in multiple disease states [7–12], providing a major source of microbes that periodically reach the lower airways. In addition, the advances in culture independent technique that allows unbiased characterization of microbes has shown that microbes are also present in the air [13–16]. Considering that we breathe approximately 4000 L of air per day, the lower airways are constantly exposed to the airborne microbiota. Also, episodic exposure to the upper airway microbiota occurs through microaspiration. With the use of these culture independent techniques that target bacterial DNA, we are now able to recognize and characterize the lower airway microbiota. Consistent with microaspiration as a main source of microbes, the lower airway microbiota is frequently enriched with oral commensals such as *Streptococcus*, *Prevotella*, *Veillonella*, *Porphyromonas* and *Rothia* [17–21]. Despite our new knowledge of the existent lower airway microbiome, our understanding of the significance of these microbes in the lower airways for multiple diseases is limited. In this review, we will focus on the current knowledge about the lower airway microbiota and its possible implications in lung cancer.

8.2 Lung Microbiome in Smoking and COPD

The lower airway microbiota is affected in several conditions that are associated with lung cancer. Smoking is a well-established risk factor for lung cancer although only 15% of smokers will develop lung cancer. In the upper airways, smoking has been associated with lower relative abundance of *Porphyromonas*, *Neisseria* and *Gemella* and higher relative abundance of *Megasphaera* spp., *Streptococcus*, *Veillonella*, *Atopobium* spp. and *Actinomyces* [21, 22]. In the lower airways however, smoking alone does not seem to cause changes in the composition of the lung microbiome [21, 23]. Two lung diseases associated with lung cancer are also associated with lower airway dysbiosis: COPD, which occurs in 15% of smokers, and emphysema, which occurs in approximately 40% of smokers. Evaluation of the microbial community in the lower airways of subjects with COPD has shown a complex and diverse microbiota [20, 24, 25]. In advanced stage COPD (GOLD 4), increased bacterial colonization and recurrent infections are associated with increased risk of exacerbations and accelerated loss of lung function [26]. Furthermore, there is reduced bacterial diversity in advanced COPD as compared with the healthy or milder cases [20]. The core

of this bacterial community may be comprised of previously unrecognized lung pathogens such as oropharyngeal and gut-associated bacterial species. As disease progresses, the lower airway microbiota is enriched with pathogens from *Gammaproteobacteria* phylum (which includes COPD associated pathogens such as *Haemophilus* and *Moraxella*). However, the early changes in lung microbiome in COPD have not been elucidated. A common finding is the enrichment of the lower airway microbiota with oral microbes, such as *Prevotella*, *Veillonella*, *Rothia*, *Porphyromonas*, and *Streptococcus* [24, 27, 28]. Importantly, enrichment of the lung microbiome with oral microbes is associated with neutrophils, lymphocytes and inflammatory cytokines [17, 18]. This is relevant since a shared feature of lung cancer, smoking, and COPD is the presence of chronic inflammation. Thus, it is possible that distinct changes in the lung microbiome may contribute to host inflammatory changes that are relevant in the pathogenesis of COPD and lung cancer.

8.3 Microbes in Lung Cancer: Lessons from Culture Dependent Methods

The idea that microbes may play a role in cancer development was introduced many centuries ago when Rudolf Virchow first noted leukocytes in neoplastic tissues and postulated that pathogens promote carcinogenesis through chronic inflammation [29]. In 1972, Schreiber et al. showed that in a nitrosamine murine model of lung cancer, there was a decreased rate of cancer development in germ-free rats compared to rats with chronic respiratory infections [30]. Chronic administration of lipopolysaccharide (LPS) has also shown to induce lung tumorigenesis in mice models [31]. Looking at epidemiological data, there is some evidence that microbes may affect lung cancer development. In a nested case-control study from the Prostate, Lung, Colorectal, and Ovarian Screening (PLCO) trial with over 77,000 subjects, antibody titers for *Chlamydia pneumoniae* were significantly increased in those with lung cancer as compared to controls [32]. In Addition, the use of antibiotics has been associated with a higher risk for developing lung cancer which suggests a potential role for dysbiosis in the pathogenesis of lung cancer [33]. Together, these examples support the theory that chronic respiratory infections may contribute to lung carcinogenesis.

8.4 Lung Cancer Microbiota: NextGen Sequencing

Modern studies using 16S rRNA gene sequencing on samples from lung cancer patients are starting to describe the lung microbiome in this disease. In a small study, the microbiome in sputum of lung cancer patients showed differences in α bacterial diversity (within sample diversity). In addition, the sputa of never smoker lung cancer subjects were enriched with *Granulicatella*, *Abiotrophia* and *Streptococcus* genera [34]. Others have found that in saliva, there was an increase in

relative abundance of *Capnocytophaga*, *Selenomonas*, and *Veillonella*, and a decrease in relative abundance of *Neisseria* species in lung cancer patients compared to controls [35]. Furthermore, in lung tissue, the genus *Thermus* was found to be more abundant in late stage (IIIB, IV) lung cancer patients, while *Legionella* was enriched in patients who developed distant metastases [36]. In another study, 16S rRNA gene sequencing was performed on bronchioalveolar lavage (BAL) fluids from patients with lung cancer and found bacteria in the lung which were commonly indigenous to the oral cavity such as *Streptococcus*, *Veillonella*, *Gemella*, *Porphyromonas*, *Olsenella*, and *Eikenella* [37]. This study suggested a possible role for micro-aspiration during lung cancer development. Nontypeable *Haemophilus influenza* (NTHi), which has been previously shown to cause COPD exacerbations, increases the risk of lung cancer development [38]. In addition, NTHi is associated with higher rates of COPD exacerbations [39, 40], airway injury and inflammation. The role of *Haemophilus* on lung cancer is also supported by experimental data. When mice with an activated K-ras mutation in their airway epithelium were exposed to chronic aerosolized NTHi lysate, the exposed mice had increased neutrophil, lymphocyte, and macrophage numbers, as well as increased numbers of lung surface tumors by 3.2 folds (156 ± 9 exposed vs. 45 ± 7 control) [41]. This type of chronic inflammation induced by lung microbiota might be the common link to lung cancer and related lung diseases such as COPD [41, 42].

8.5 Microbial Regulation of Host Inflammation and Host Immune Surveillance with Possible Effects on Lung Cancer Pathogenesis

Carcinogenesis has commonly been divided into four stages: initiation, promotion, malignant transformation, and tumor progression. Epithelial cells of the airway mucosa are constantly exposed to environmental toxins and microorganisms that may affect each stage of carcinogenesis. In addition, there is better understanding of the role of inflammation and immunological surveillance on the development and treatment response in lung cancer. Both host inflammation and host immune surveillance are susceptible to microbial regulation.

While most studies have thus far focused on describing the composition of the lung microbiota in health and disease, recent publications have started to shed light on the interactions between the microbes and the host in the lower airways. A common pattern found in multiple studies is the enrichment of the lower airways with oral taxa. We have adopted the term supraglottic predominant taxa pneumotype (pneumotypeSPT) to describe this pattern. Our initial investigations have shown that this pattern occurs in both smokers and non-smokers and is associated with increased neutrophils and lymphocytes [17]. We then extended these observations in a multicenter trial and showed that pneumotypeSPT was associated with a distinct metabolic and immunological profile characterized by a Th17 phenotype as well as a contra-regulatory mechanism (e.g. blunting of TLR4 responses) [18].

HIV is also a risk factor for lung cancer even after immune reconstitution with anti-retroviral therapy [43]. Evaluation of the lung microbiome in HIV has shown distinct features with high frequency of *Tropheryma whipplei* [44] as well as enrichment of lower airway microbiota with oral taxa in subjects with moderate immunosuppression (CD4 ~260 μL^{-1}) that persists after anti-retroviral therapy [45]. Furthermore, in HIV-infected individuals with pneumonia, presence of a lower airway microbiota enriched with oral taxa, such as *Prevotella* or *Streptococcus*, was associated with distinct local and systemic host immune responses as well as worse rates of mortality that could not be explained by the pathogen isolated based on culture technique [46]. These studies suggest that lower airway dysbiosis contributes to the lower airway immune phenotype, potentially influencing cancer pathogenesis.

The molecular mechanisms for these associations are still not clear. We recently showed that short chain fatty acids (SCFA), intermediate products of microbial anaerobic metabolism, can be found in the lower airways and that increased systemic levels are associated with a higher risk for pathogen acquisition [47]. Importantly, increased levels of SCFA in the lower airways are associated with enrichment of the lower airway microbiota with oral anaerobes, supporting the idea that this distinct pneumotype is metabolically active. Biofilm formation potential and the ability to generate a hypoxic niche are affected by multiple disease states and is increased by smoke [48]. Biofilms provide the microbial environment needed for anaerobes to use fermentation as a source of energy (Fig. 8.1). SCFAs are important for the expression of forkhead transcription factors, such as FoxP3 on $CD4^+$ lymphocytes, leading to a regulatory T cell phenotype [49]. This effect may

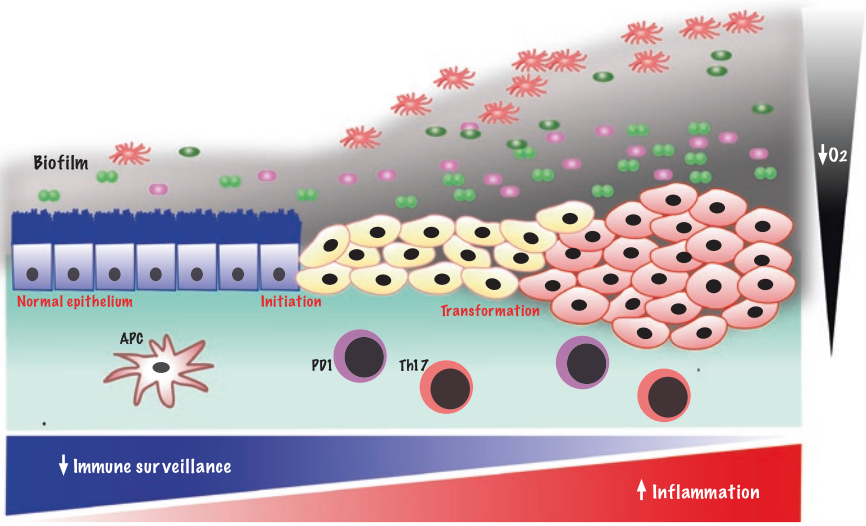

Fig. 8.1 Conceptual model of lower airway microbiota and host interaction with relevance for cancer pathogenesis

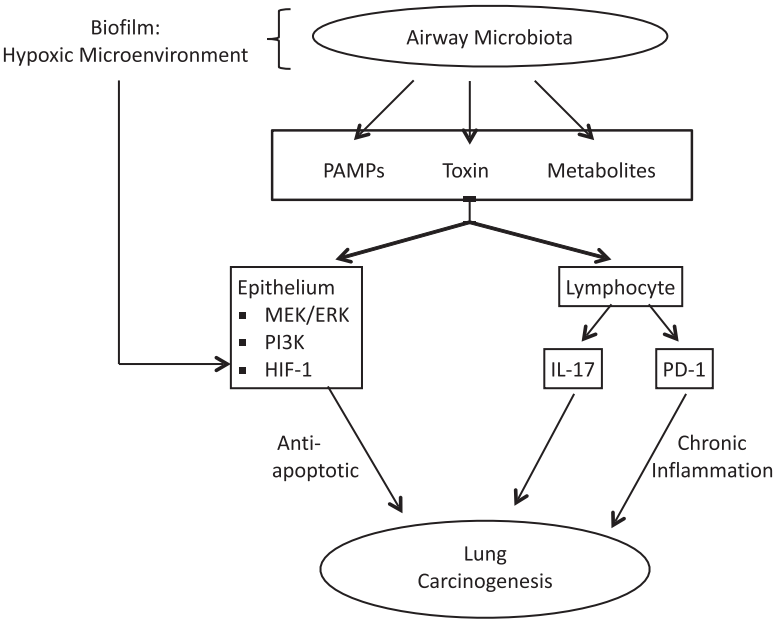

Fig. 8.2 Microbial triggers and lung carcinogenesis

be important in immune tolerance. In the lung, increased SCFA levels inhibited IFN-γ production, raising the possibility that these anaerobic fermentation end products induce T cell exhaustion and susceptibility to pathogens [47].

The described microbe-host interactions may occur and be relevant in cancer carcinogenesis (Fig. 8.2). For example, IFNγ and IL17 play a significant role in lung cancer formation. The Th17 cell phenotype plays a key role in activating the host immune defense against microbes at mucosae sites. Newer evidence shows that interleukin 17, the pro-inflammatory cytokine produced by activated Th17 cells, plays an active role in inflammation-associated carcinogenesis [50–53]. Th17 cells are frequently observed in non-small cell lung cancer by immunohistochemistry [54]. A high level of IL-17 was associated with smoking status, TNM stage, lymphatic vessel density, and overall survival and disease-free survival. Evidence also suggests that IL-17 expression influences angiogenesis and lymphangiogenesis to promote tumor progression [53]. Th17 cell differentiation is an important host immune response at mucosal sites and helps protect against extracellular bacterial and fungal pathogens. When microbes or microbe-associated molecular pattern products are recognized by Toll-like receptors, downstream activation of nuclear factor-κB (NF-κB) and signal transducer and activator of transcription 3 (STAT3) leads to Th17 cell differentiation [55, 56]. Breakdown of tight junctions in epithelial cells may lead to microbial products entering the tumor microenvironment. In colorectal cancer this event was shown to activate IL-23-producing myeloid cells which eventually induced IL-17 production [57]. Experimental models showed

that chronic intranasal injection of lipopolysaccharide (LPS), a glycolipid component of gram-negative bacterial membranes, into A/J mice pre-treated with 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) induced lung tumor development by increasing macrophage recruitment and activating Akt, NF-κB, and STAT3 signaling pathways [31]. Akt and STAT3 signaling pathways are associated with altering the Treg/Th17 balance [55, 58, 59] by increasing Th17 differentiation [60–62].

MAPK-ERK and PI3K/Akt signaling pathways consist of kinase cascades which are well studied in carcinogenesis. These pathways play important roles in regulating cell proliferation, survival, and differentiation. Exposure to opportunistic pathogens activates the RAS-MAPK-ERK signaling pathway which in turn can slow cell cycle development [63]. Microbial activation of this pathway has been shown to have a pathogenic role in other epithelial cancers. For example, cytotoxin-associated gene A produced by *Helicobacter pylori* has been shown to interact with host proteins to activate the MEK/ERK pathway [64]. Closely associated with the MAPK-ERK signaling pathway is the PI3K/Akt signaling pathway. PI3K/Akt is also involved in regulation of cell proliferation, survival, differentiation, and cell invasion. Its activation in bronchial airway epithelium has been shown to be an early and reversible event in lung tumor development [65], and its deregulation has been associated with advanced stage lung cancer [66]. Somatic PIK3CA mutation has been found in 3-10% of squamous cell lung cancers [67] and 1–3% in lung adenocarcinomas [68]. *Porphyromonas* is an oral commensal previously associated with oral squamous cell carcinoma [69] and pancreatic cancer [70]. It is enriched in the lower airways in subjects with pneumotypeSPT. Importantly, *Porphyromonas* has been found to activate the ERK pathway as well as the PI3K/Akt signaling pathway in the oral mucosa [71]. In lung cancer patients, enrichment of the lung airway microbiota with oral commensal is associated with up-regulation of ERK/PI4k signaling pathways [001]; while *Acidovorax* is associated with TP53 mutations in squamous cell lung cancer [002]. Given the relevance of this pathway in lung cancer pathogenesis, future work should evaluate whether this bacteria contributes to the deregulation of this pathway in the lower airways.

While increased Th17 driven inflammation has been associated with lung cancer, deficiency of IL-17 may also have a pathogenic role. Antibiotic-treated mice exhibited larger tumor size with increased number of tumor foci compared to untreated mice [72]. These mice were found to have defective γδT cells and reduced IL-17 levels. γδT cells are predominately found in epithelial linings of lung, tongue, genital tract, and liver, and they are important in the innate immune response by producing IFNγ and IL-17 cytokines. The reduction of IL-17 may lead to dysfunctional microbial surveillance in the airway mucosa favoring dysbiosis with potential impact on lung cancer pathogenesis.

Cytotoxicity mediated by effector T cells is an important defense mechanism against malignant transformation of cells. Cytotoxicity is highly regulated by IFN-γ. The decreased IFN-γ likely contributes to a suppressive pro-tumor microenvironment and the survival of cancer cells [73]. This cytokine inhibits angiogenesis and cellular proliferation and promotes apoptosis of cancer cells.

Microbial products such as SCFA might affect IFN-γ release by both CD4 and CD8 T cells [47], likely affecting cytotoxicity against malignant cells. Interestingly, microbes can also cause tumor promotion and malignant transformation by inducing proliferation pathways and inhibiting apoptotic pathways. Hinnebusch et al. showed that bacterial metabolites, such as short chain fatty acids (e.g., butyrate), induced histone hyperacetylation, increased cell apoptosis and inhibited cell proliferation in colon cancer [74]. Given the prevalence of oral anaerobes in the lower airways frequently described in multiple lung microbiome studies, future investigations should evaluate the role of the hypoxic environment through biofilms in the lower airways associated with lung cancer.

In addition, LPS has been demonstrated to directly induce hypoxia inducible factor (HIF) activation by increasing HIF-1α protein expression through translational and transcriptional dependent pathways in macrophages [75]. Numerous bacterial species, including group A *Streptococcus*, *Staphylococcus aureus*, *Salmonella typhimurium* and *P. aeruginosa*, have been shown to have profound host innate immune response by increasing HIF levels in macrophages and neutrophils [76]. HIF-1α, a transcription regulator that is stabilized under hypoxic stress [77, 78], is also a promoter of Th17 differentiation [79]. Importantly, HIF-1α is found to be up-regulated in lung tumors [80], and is involved in promoting lung tumor growth through apoptosis inhibition [81] and VEGF-dependent mechanisms [82]. In addition, HIF-1α expression correlates with worse clinical prognosis [80]. Airway biofilm have long been observed in patients with chronic pulmonary diseases. The biofilm formation on mucosal membranes provides a structural niche for microbes and allows them to survive under poor nutrient conditions. The biofilm microenvironment within the pulmonary airways is a perfect reservoir for anaerobic bacterial growth and development of a local hypoxic micro-environment. Evidence suggests that tumor cells and microbes (biofilm) reside in a hypoxic microenvironment and can up-regulate HIF-1α expression [81, 83].

Other mechanisms by which microbes can promote carcinogenesis include (1) direct damage of mammalian DNA by microbial byproducts such as Cytolethal distending toxin (CDT) and Colibactin [84, 85], and (2) induce chronic inflammation through reactive oxygen species (ROS)-mediated genotoxicity [86]. While not fully address in this review, it is also important to note that cause-effect relationship is still unclear, and that lung cancer may likely disrupt the local microbiota composition and systemic host immunity, which may exert significant selection pressure on the microbiota and possibly affect treatment response (see below).

8.6 Microbial Effect on Cancer Immunotherapy

Checkpoint inhibitor immunotherapy is an effective treatment for many solid tumors, including lung cancer. There is evidence to suggest that the microbiota affects expression of immune checkpoint molecules. Chronic antigen stimulation of T cells lead to exhaustion of effector T cells characterized by loss of proliferative

potential, cytokine production, and cytotoxicity [87]. This phenotype is characterized by accumulated expression of what has been classified as immune checkpoint molecules, such as programmed death 1 (PD-1). Programmed death-ligand 1 (PD-L1), a molecule that can be found in lung cancer and binds to PD1, exacerbates T cell dysfunction. In a mouse model, Gollwitzer et al. showed that early life constitution of lower airway microbiota contributed to PD-L1 expression on CD 11b+ dendritic cells [88]. Changes in PD-L1 expression also coincided with lower peak levels of regulatory T cells. This change in lung microbiota appears to influence maturation of the immune system and disruption during this critical phase may affect adulthood immunity. Expression of immune checkpoint molecules are also affected after Chlamydia respiratory infections in early life with increases in PD-l and PD-L1 mRNA expression, increases in the number of PD-1+ and PD-L1+ monocytes, myeloid cells, dendritic cells, and CD4+ or CD8+ T cells [89]. In addition, this seems to have a long-term consequence on pulmonary function. Thus, modulation of the microbiome can enhance antitumor immunity and augment effects of the PD-L1 checkpoint blockade in cancer therapy [90]. Similar results were seen in antitumor effects of CTLA-4 monoclonal antibodies when the gut microbiome was modified [91]. Recent data also suggest that resistance to anti-CTLA-4 therapy may be due to loss of IFN-γ- signaling, and more specifically, due to decreased INF-γ receptor 1 (IFNGR1) expression [92]. It is well known that INF-γ is an important cytokine for host defense against infection by viral and microbial pathogens. Therefore, it is possible that certain microbes may increase the anti-tumor effect of CTLA-4 by inducing a high INF-γ-signaling or up-regulating IFNGR1 expression. Prospective studies in 112 metastatic melanoma patients demonstrated those who were responders to anti-PD-1 therapy or with prolonged progress-free survival had higher α diversity in fecal microbiome and were enriched with *Clostridiales*/*Rumin ococcaceae* [93]. In 140 patients diagnosed with non-small cell lung cancer (NSCLC), antibiotic use around the time of anti-PD-1 therapy was a predictor of resistance to PD-1 blockade, which was validated in a separate cohort of 239 NSCLC patients [94]. Also in this study, mice that were transplanted with feces from anti-PD-1 “responder” patients had tumor growth delay and upregulation of PD-L1 after PD-1 blockade. While these studies focused on gut microbiome, further research on the effect of lung microbiome on immune checkpoint blockade antibodies may yield important information regarding therapies in lung cancer patients.

8.7 Other Lung Cancer

There is limited data on airway microbiome and any other lung malignancy, with the exception of malignant pleural mesothelioma, a rare type of lung cancer in the pleura associated with asbestos exposure. Currently, it is unclear how asbestos fibers deposited in the lung translocate to the pleural space. It has been proposed

that certain secreted proteins by airway microbiota can create pores, which allow asbestos to reach the visceral pleura after inhalation [95]. Thus, microbial derived products can indirectly affect the pathogenesis of mesothelioma.

8.8 Lung Microbiome and Cancer: What Can We Expect from Future Investigations?

Lung cancer screening remains an area where novel biomarkers are needed. Despite the evaluation of significant numbers of proposed biomarkers, there is currently none available for routine use. Identification of distinct microbiota associated with lung cancer could potentially represent a novel biomarker for lung cancer diagnosis or prognosis. The few studies performed that have identified groups of bacteria associated with lung cancer [34, 36, 37] are limited, small in sample size, and lack a validation cohort. For example, a proposed saliva-based microbiota biomarker had an area under the receiver operating characteristic curve value of 0.86 (with 84% sensitivity and 87% specificity) for squamous cell carcinoma and a value of 0.80 for lung adenocarcinoma [35]. And more recently, it was shown that an increase in relative abundance of *Veillonella* and *Megasphaera* in BAL had area under the curve of 0.888 in predicting presence of lung cancer [96]. In addition, metagenomic sequencing and metabolomic profiling [97] of sputum microbiome [98] may help differentiate those subjects with and without lung cancer. These prior investigations provide the initial insights into the use of microbiomic approaches as potential biomarkers for lung cancer.

In conclusion, we are in the early stages of understanding the role of the lower airway microbiota in inflammatory diseases. Even less is known about its role in carcinogenesis. However, there is significant mounting evidence of the plausibility of this association with potential therapeutic implications. For example, animal models show that checkpoint inhibitor's antitumor activity can be modulated through changing the microbiome [90, 91]. In addition, in animal models, lung cancer size, number of tumor nodule, and survival rate can be influenced with antibiotic treatment [72]. More is needed to elicit the cause-effect relationship between lung cancer and microbiome. This data highlight that strategies aimed at modifying the composition of the lung microbiota (e.g. probiotics/prebiotics, antibiotics, diet, mucosal microbiota transplantation) could have a potential therapeutic role in lung cancer.

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Chapter 9
Infectious Agents Associated with Mesothelioma

Nguyen Son Lam, Nguyen Van Tho, Tran Dinh Thanh, and Yasutaka Nakano

Abstract Malignant mesothelioma is a rare but fatal disease which arises from the epithelial lining of the pleura, peritoneum, pericardium, and tunica vaginalis. Malignant pleural mesothelioma (MPM) is the most common form. The global incidence of MPM has risen steadily over the past decade and is predicted to peak in 2020. The mechanism of carcinogenesis in MPM is multifactorial. A history of long-term exposure to asbestos is the established cause of MPM. Cytolysins such as Pneumolysin, Streptolysin O, Intermedilysin, Mitilysin, and Lectinolysin secreted by the airways microbiota may create pores through which asbestos can pass through the airways, reach the visceral pleura and cause MPM. However, MPM may result from other factors such as genetics, erionite, chest wall radiation, and simian virus 40 (SV40) that may work alone or in combination. The roles of SV40 in malignant mesothelioma is still controversial. More studies are needed to explain the wide disparity in the prevalence of SV40 in mesothelioma tissues reported by different laboratories or regions. In this chapter we discuss about how infectious agents may be associated with malignant mesothelioma.

Keywords Immunohistochemistry · Malignant mesothelioma · Pleural · Simian virus 40 · Tumor · Viral carcinogenesis · Viral infection

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9.1 Introduction about Malignant Mesothelioma

9.1.1 Epidemiology of Malignant Mesothelioma

Malignant mesothelioma was first recorded in 1870 and the relation between malignant mesothelioma and asbestos exposure was established in 1960 in South African [1]. Malignant mesothelioma is a rare but fatal disease which arises from the epithelial lining of the pleura, peritoneum, pericardium, and tunica vaginalis. Malignant pleural mesothelioma (MPM) is the most common form, accounting for 80–90% malignant mesothelioma [2]. Global incidence of MPM has risen steadily over the past decade and is predicted to peak in 2020 [3]. The incidence of malignant mesothelioma is usually underestimated, especially in developing countries. An estimate based on 1994–2008 database suggested an average of 14,200 cases worldwide each year [4]. About 3000 new cases of mesothelioma are diagnosed in the US each year, more often in men, in those aged 65 years and older, and in whites [5].

The mechanism of carcinogenesis in MPM is multifactorial. A history of heavy and long-term exposure to asbestos is the established cause of MPM [6]. However, a history of asbestos exposure have not been found in 20–60% patients with MPM [7]. In these patients, MPM may result from other factors such as genetics, erionite (a mineral found in the rocks of Turkey), chest wall radiation, and simian virus 40 (SV40) that may work alone or in combination [8]. Whatever the etiology, the clinical scenario of MPM is the same.

9.1.2 Histological Sub-Types of Malignant Pleural Mesothelioma

There are four main histological sub-types of MPM: epithelioid (the most common sub-type), sarcomatoid, biphasic, and desmoplastic. A recent study showed that among 45 patients with MPM, 23 (51%) was epithelioid, 7 (16%) biphasic, 6 (13%) sarcomatoid, 4 (9%) desmoplastic, 4 (9%) well-differentiated papillary, and 1 (2%) anaplastic subtype [9]. The sarcomatoid sub-type is associated with the worst outcomes, with a median survival of just 4 months. In contrast, the epithelioid sub-type has the most favourable prognosis with a median survival of 13.1 months [10]. Favorable predictors of overall survival were younger age, female, epithelioid sub-type, well differentiated grade, surgically or radiationally cancer-directed therapy [11]. The median overall survival of patients with MPM in the United State was 8 months [5].

9.1.3 Symptoms of Malignant Pleural Mesothelioma

The clinical onset of MPM is insidious and patients usually have non-specific symptoms. The majority of patients with MPM present with breathlessness, chest pain or both [12]. Dyspnea is the most common in MPM; the level of dyspnea increases

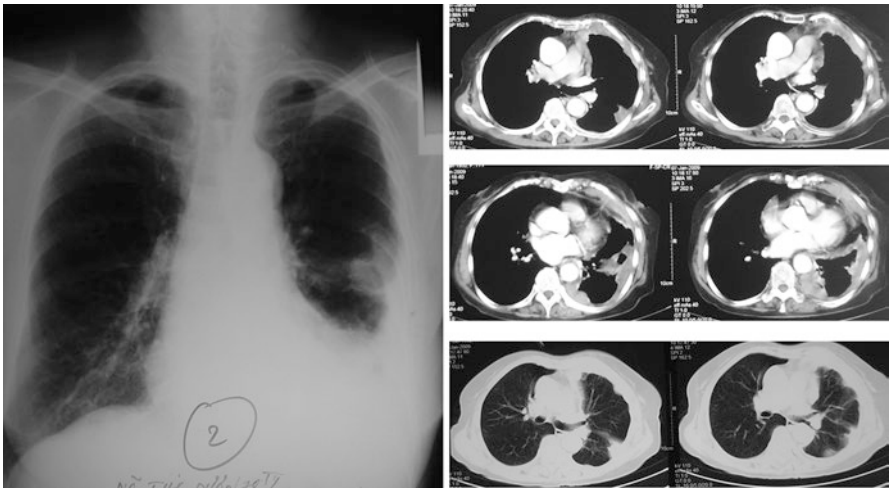

Fig. 9.1 Chest X-ray and CT images of a 77-year-old female patient with pleural malignant mesothelioma. The chest X-ray shows mild left pleural effusion accompanied with mild volume reduction of the left hemithorax. The chest CT images in mediastinal window show mild left pleural effusion and localized pleural thickenings which cause mild volume reduction of the left hemithorax. The chest CT images in parenchymal window show rind-like encasement of the left hemithorax

over time. The pleural effusion is mainly unilateral (95%), especially on the right lung (60%). Patients may present as chest pain, which can be caused by the pleural effusion or the tumor. When the tumor invades the chest wall or ribs, the severity of chest pain increases [6].

Other symptoms of MPM include fatigue, anorexia, weight loss, sweats and malaise which result from circulating cytokines, released by both the tumor and host response [12]. Cough, haemoptysis and lymphadenopathy are less common in MPM than in bronchogenic tumors.

Pleural effusion can be detected by chest X-ray. Most patients with MPM present with large pleural effusion on chest X-ray [1]. Chest CT is more sensitive than chest X-ray in detecting other signs of MPM such as localized effusion, diffuse pleural thickening, rind-like encasement of the entire lung, pleural focal masses (Fig. 9.1). CT is also useful for detecting hilar or mediastinal lymph node enlargement, and mediastinal or chest wall invasion [13].

9.1.4 Diagnosis of Malignant Pleural Mesothelioma

Diagnosing MPM is challenging because cytological evaluation yield of pleural fluid is low with a sensitivity of 26%. Biopsies are usually required to confirm the diagnosis and identify the histological sub-type. Biopsies can be obtained by using a blind Abrams needle method, or under direct vision at thoracoscopy, either as a

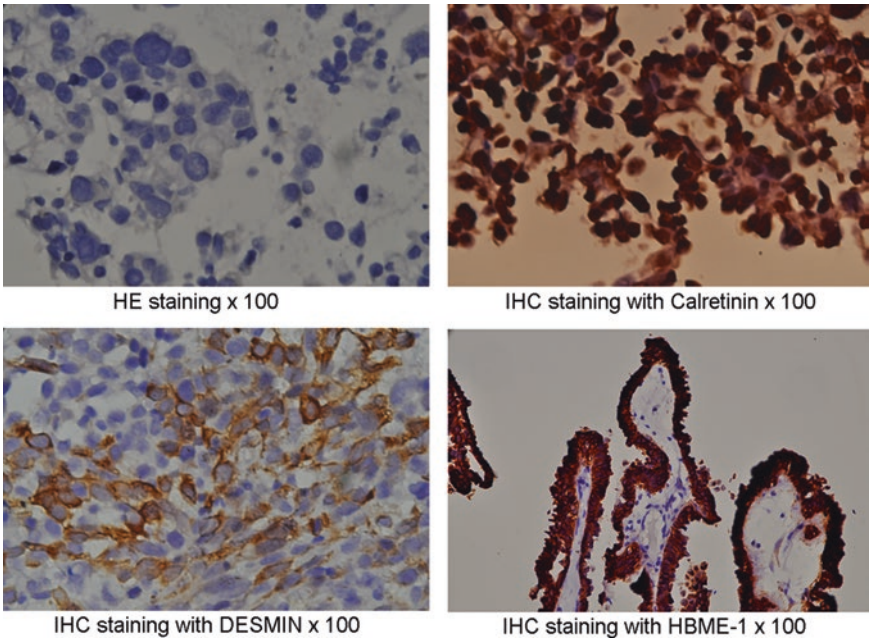

Fig. 9.2 Hematoxylin-eosin (HE) and immunohistochemical (IHC) staining to diagnose malignant mesothelioma. Left upper image: Epithelioid mesothelioma stained by hematoxylin-eosin method (original magnification ×100). Right upper image: Epithelioid mesothelioma stained by IHC method which is positive with Calretinin (original magnification ×100). Left lower image: Sarcomatoid mesothelioma stained by IHC which is positive with DESMIN (original magnification ×100). Right lower image: Well-differentiated papillary mesothelioma stained by IHC which is positive with HBME-1 (original magnification ×100)

medical thoracoscopy or as a video-assisted thoracoscopic surgery [14]. Diagnosis is achieved by needle biopsy in 21% and by thoracoscopy in 98% of patients [15].

Most patients with MPM are diagnosed definitively based on the histological examination of pleural specimens by using hematoxylin-eosin and immunohistochemical staining (Fig. 9.2) [16, 17]. Immunohistochemical panels are integral to the diagnosis of MPM, but the exact makeup of panels employed is dependent on the differential diagnosis and on the antibodies available in a given laboratory. Depending on the morphology, immunohistochemical panels should contain both positive and negative markers for malignant mesothelioma and for lesions considered in the differential diagnosis. Immunohistochemical markers should have either sensitivity or specificity greater than 80% for the lesions in question [18].

Four positive markers including Calretinin, DESMIN, HBME-1, and WT-1 have been used to definitively diagnose MPM (Fig. 9.2). Different negative markers have been used to rule out other cancers metastasized to the pleura such as CK7, CEA, TTF-1, and EGFR for adenocarcinoma; NSE, Synaptophysin, and MOC-31 for small cell lung cancer; LCA, CD3, CD20, CD30, CD68, and Myeloperoxidase for lymphoma and leukemia [18].

9.1.5 Management of Malignant Pleural Mesothelioma

There is no curative treatment for MPM. Systemic treatment options include chemotherapy (pemetrexed and cisplatin/carboplatin), targeted therapy (bevacizumab, an anti-VEGF monoclonal antibody) and radiotherapy, delivered separately or as part of multimodality treatment. Surgery (pleurectomy and decortication) is controversial and limited to patients with early stage and good functional status. Palliative care and symptom management are essential and the control of pleural effusions is an important factor.

A number of novel therapeutic agents are under investigation, and may provide further treatment options for MPM in the future [12]. Mesothelin is a cell surface glycoprotein highly expressed in MPM. Its expression induced matrix metalloproteinase secretion and cell invasion and it was validated as a potential target with both tumor vaccines and antibody-based approaches [19].

Amatuximab is a chimeric monoclonal antibody to mesothelin. It elicits antibody-dependent cellular cytotoxicity against mesothelin-expressing tumor cells and inhibits heterotypic adhesion of mesothelin-positive tumor cells to CA125-expressing tumor cells. A phase II clinical trial of amatuximab with pemetrexed and cisplatin in patients with unresectable MPM showed that this treatment was safe and well tolerated. Although there was no improvement in progression-free survival at 6 months (51%), the median overall survival (14.8 months) was superior to historical controls [20]. CRS-207 is live, attenuated, double-deleted Listeria monocytogenes engineered to express the tumor-associated antigen mesothelin, activating innate and adaptive immunity. The combination of CRS-207 and chemotherapy may act synergistically to alter the tumor microenvironment to potentiate immune-mediated killing. A phase 1b trial in 38 patients with unresectable MPM showed that CRS-207 has been well tolerated. In combination with pemetrexed plus cisplatin, infusions of CRS-207 resulted in a 59% rate of partial response and a median progression-free survival of 8.5 months [21].

9.2 Possible Mechanisms of Mesothelioma Development Associated with Microbiome

In the parietal pleura, where MPM predominantly arises, only ultra-thin and mostly ultra-short fibers of asbestos have been observed. There are two main theories regarding the pathways through which the inhaled fibers reach the pleural surface. Asbestos can either reach the pleural cavity in a mechanical fashion by their extrusion from the alveoli and the lung parenchyma passing through the visceral pleura or through being absorbed by the lung lymphatic system that results in the dissemination throughout the body [22]. For the first pathway, Magouliotis et al. proposed that toxins such as cholesterol-dependent cytolysins (CDC) secreted by the airways microbiota create pores through which asbestos can pass through and reach the visceral pleura [23]. CDCs' action depends on the cholesterol component of the cell

membranes. Therefore, the secretion of a CDC in an individual exposed to asbestos could potentially create the pathway through which an ultrathin fiber can escape the airways and penetrate deeper. The effect of these toxins on the plasma membranes lead to the production of pores with an average diameter of 35–50 nm [24]. The diameter of pores should be bigger than the lower limit of the width of asbestos fibers and the physical flora of the anatomical area near the pleura should contain microorganisms that produce these certain toxins. In fact, Pneumolysin (PLY), Streptolysin O (SLO), Intermedilysin (ILY), Mitilysin (MLY) and Lectinolysin (LLY) are the five main CDC toxins that could take part in the proposed mechanism and all of them are produced by species of the Streptococcaceae family, S. pneumoniae, S. pyogenes, Streptococcus intermedius and S. mitis [23].

Vascular endothelial growth factor (VEGF) plays a key role in tumorigenesis and progression of malignant mesothelioma. Mesothelial cells are unique in preventing fibrosis and adhesive lesions in the body cavities including the pleura, pericardium and peritoneal cavity. Mesothelial cells express VEGF and specific VEGF receptors. VEGF is a mitogen for endothelial cells and enhances vascular permeability [25]. In addition, it also enhances permeability in the mesothelial monolayer. The formation of pleural effusions generally involves the migration of cells and plasma from the systemic circulation to the pleural space across the vascular and mesothelial barriers [26]. VEGF receptors include Toll-like receptor 3 (TLR3), RIG-I and MDA5. TLR3 recognizes dsRNA of viral origin. Activation of TLRs leads to increase of VEGF synthesis [27]. Wornle et al. demonstrated that activation of mesothelial viral receptors leads to a time- and dose-dependent increase of mesothelial VEGF synthesis [28]. This observation could explain how viral infections can lead to pleural effusions and contribute to tumorigenesis and proliferation of malignant mesothelioma.

9.3 The Relation Between Malignant Mesothelioma and Simian Virus 40

9.3.1 Simian Virus 40

Simian virus 40 is a non-enveloped DNA virus and classified as a member of the polyomavirus family, based on the size (about 40 nm in diameter) and morphology of its icosahedral capsid (Fig. 9.3) and on the size of its double-stranded DNA genome [29, 30]. Its genome consists of a single circular double stranded DNA molecule and can be divided into three distinct regions—early, late and regulatory. The early region is expressed soon after entrance into the host cell, while the late region is expressed efficiently only after successful viral DNA replication has begun and it encodes for the capsid proteins (Fig. 9.4). Its closest relatives are two polyomaviruses recovered from humans, JC virus (JCV) and BK virus (BKV). They have shared about 69% genomic similarity at the nucleotide level, with the lowest similarity in the regulatory region sequences. The large T antigens (Tag) of the

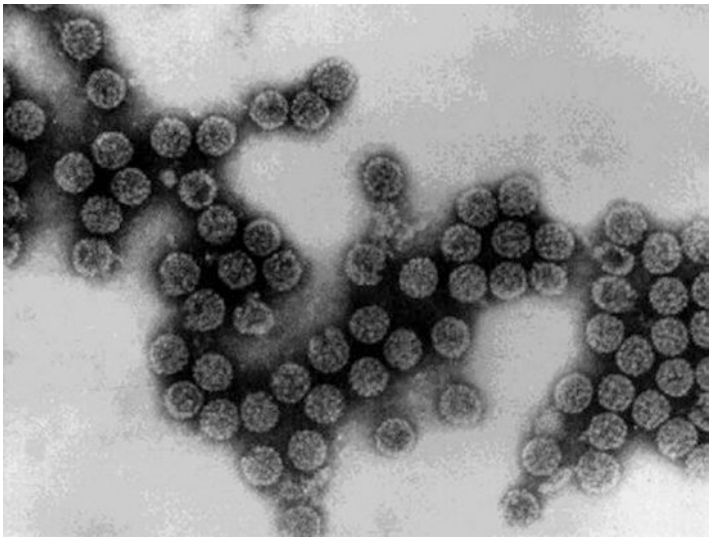

Fig. 9.3 Negative stained Transmission Electron Micrograph (TEM) shows some of the morphological features displayed by a number of Simian virus 40 virions (photo by Dr. E. Palmer, Center for Disease Control, GA, USA; no copyright restrictions under Public Domain—Property of the United States federal government)

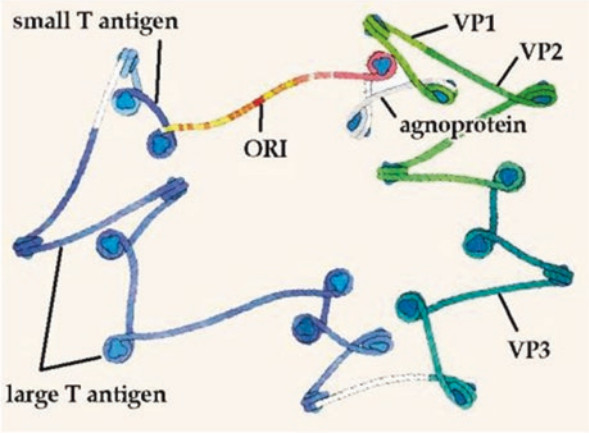

Fig. 9.4 Structural view of the 5243 nucleotide SV40 genome with its characteristic nucleosomes. Blue highlights the early region, while the late region is green. Yellow and red denote the regulatory region of the viral genome (modified from D.S. Goodsell. Simian Virus 40—November 2003 Molecule of the Month. Research Collaboratory for Structural Bioinformatics (RCSB) Protein Data Bank; no copyright restrictions under Public Domain)

polyomaviruses have about 75% amino acid identity [31]. Although they are closely related, they can be distinguished at the level of DNA and protein and can be distinguished by neutralizing serum and hemagglutination inhibition tests. Humans usually have JCV and/or BKV infection, so it is necessary to use specific viral reagents to detect the presence of SV40 in human tissues [32–35].

Common laboratory strains of SV40 were isolated in 1960 from contaminated vaccines or from cultured kidney cells derived from a control group of brown, green, or patas monkeys. Although there is only one known serotype of SV40, different virus strains persist and can be distinguished by changes in the structure of the virus and the designated area of the nucleotide sequence C terminus extreme Tag gene [32, 36]. Distinct nucleotides were used to demonstrate that the viral sequences involving human beings were not resulted from laboratory contamination [18, 32, 34].

9.3.2 Epidemiology of SV40 Infection in Humans

Natural infection by SV40 in humans was rare, restricted to people living in contact with monkeys, the natural hosts of the virus, such as inhabitants of Indian villages located close to the jungle, and persons attending to monkeys in zoos and animal facilities [33, 37]. SV40 can naturally infect rhesus monkey renal cells and is now widespread among the human population. The modes by which the virus has been transferred from monkeys to humans are uncertain, but it may be that the majority of this transmission might had occurred from 1954 to 1963 when hundreds of millions of people in the United States, Canada, Europe, Asia and Africa had been vaccinated with both inactivated and live polio vaccine contaminated with SV40. Barbanti-Brodano et al. showed that people who were vaccinated with the polio vaccine contaminated with SV40 shed the virus in feces for at least 5 weeks after vaccination [32]. This observation suggested that SV40 may be transferred from recipients of contaminated polio vaccine by orofecal route, and raise the possibility that, although human cells are less sensitive to SV40 replication compared with monkey cells, SV40 will spread among people due to horizontal transmission [35, 37].

The history of SV40 has been interwoven with the development of the polio vaccine. Both inactivated and live-attenuated forms of polio vaccine, as well as a number of other viral vaccines, have been prepared in primary cultures of rhesus monkey kidney cells, some of which was naturally infected with SV40 [32]. The contaminating virus escaped detection until African green monkey kidney cells were used and the presence of the virus was recognized by the development of cytoplasmic vacuolizations [30, 34, 36].

All polio vaccines were SV40 free in the United States after 1961 but SV40-contaminated polio vaccines might still be available in several countries after 1961 [38]. When using polymerase chain reaction method (PCR) to test vaccine samples from 13 countries and the World Health Organization seeds, Cutrone et al. found that all the vaccines were SV40 free, except for vaccines from a major eastern European manufacturer. These SV40-contaminated vaccines were produced from

1960s to 1978 and were used throughout the world. The procedure used by this manufacturer to inactivate SV40 in oral poliovirus vaccine seed stocks based on heat inactivation in the presence of $MgCl_2$ did not completely inactivate SV40 [38]. These findings explain possible geographic differences in SV40 exposure and different percentages of SV40-positive tumors detected in some laboratories.

9.3.3 Evidence Supporting Possible Roles of SV40 in Malignant Mesothelioma

Substantial evidence supports a role for SV40 in mesothelioma pathogenesis. SV40 is present in human mesotheliomas, where it is specifically found in the tumor cells and not in the normal surrounding tissues [30]. Mechanistic experiments in human mesothelial cells and animal experiments support a pathogenic role of SV40 in the pathogenesis of some mesotheliomas, including as a co-factor with asbestos [39].

SV40 plays causal role in the induction of malignant mesothelioma in hamsters. In an experiment, 100% Syrian hamsters developed mesotheliomas when wild type SV40 was injected into the pleural space. When SV40 was injected via the intracardiac or intraperitoneal routes, more than 50% of hamsters developed mesothelial tumors [40]. The possibility of mesothelioma induced by SV40 depends on the route of virus injection and types of mesothelial cells.

Why is SV40, not human polyomaviruses JCV and BKV, a carcinogen in malignant mesothelioma? Carbone et al. performed another experiment by culturing four types of human mesothelial cell lines with SV40, JCV, and BKV. They found that JCV did not infect human mesothelial cells. BKV and SV40 infected mesothelial cells, expressed viral oncoproteins, and caused similar alterations of key cell regulatory genes. BKV replicated faster than SV40 and caused mesothelial cell lysis, not cellular transformation. SV40 did not lyse mesothelial cells and caused a high rate of transformation [41].

Experiments in hamsters showed strong cocarcinogenesis between asbestos and SV40. SV40 did not cause malignant mesothelioma, asbestos caused malignant mesothelioma in 20% of hamsters, and asbestos and SV40 together caused malignant mesothelioma in 90% of hamsters. These findings suggested that significantly lower amounts of asbestos were sufficient to cause malignant mesothelioma in animals infected with SV40 [42].

To test the hypothesis that SV40 may contribute to the onset of malignant mesothelioma, Comar et al. conducted a molecular epidemiological study on a series of malignant mesothelioma patients from an area in north-eastern Italy hyperendemic for malignant pleural mesothelioma. They collected 63 mesothelioma samples from incidence cases of patients diagnosed with malignant pleural mesothelioma in the period 2009–2010. SV40 sequence detection and quantification was performed by specific real-time PCR. SV40 was detected in 22% of malignant mesothelioma tumors, with a low viral load. In SV40-positive patients, a threefold increased risk of asbestos exposure was observed, more evident in females (OR 4.32) than in males (OR 1.20) [43].

These findings implied that although asbestos was considered the main risk factor in malignant mesothelioma onset, a role for SV40 could be hypothesized [43].

Jin et al. performed a retrospective study on 18 autopsy samples of Japanese patients with pleural malignant mesothelioma from five hospitals in Japan. In order to detect SV40, PCR for SV40 Tag genome was undertaken following DNA sequence analysis and immunohistochemical staining for SV40 Tag. They found that SV40 Tag genome was detected in 8 amongst 19 malignant mesothelioma cases by one primer PCR. No immunopositive staining for SV40 Tag was found in any of the samples [44]. This study showed that SV40 genome was present in a subset of Japanese malignant mesothelioma patients who were unlikely to have received a contaminated polio vaccine based on their age.

A recent study was conducted to investigate the proportion of SV40 present in the histological specimens of the Vietnamese patients with MPM. Nine (20%) out of 45 patients with MPM in Vietnam were positive with SV40 Tag expression in their histological specimens [9]. This finding implied that SV40 could be another potential cause of MPM in Vietnam and this potential relation needs further investigation.

9.3.4 Potential Mechanism for SV40 to Cause Malignant Mesothelioma

Mesothelial cells of hamsters are more sensitive to SV40 compared to those of humans [45]. Cellular infection by SV40 is divided into several steps: an attachment phase followed by entry of the virus, transport in the cell, then a loss of the protein coating, the production of viral proteins and finally virus replication. The latter step generally induces cell lysis. In mesothelial cells, it has been hypothesized that this last step is limited, and this may be the reason why mesothelial cells are more susceptible to virus infection (Fig. 9.5) [29].

SV40 can transform human mesothelial cells with a “hit and run” type of mechanism. When exposed to SV40, most human mesothelial cells are infected, compared to about 20% of fibroblasts. Then most SV40-infected human mesothelial cells survive infection. When SV40 infects human mesothelial, it replicates; however, fewer viral particles are produced than in human fibroblasts and, therefore, cell lysis is infrequent. Expression of the SV40 tumor antigens (Tag and the small t antigen, tag) in 100% of the infected cells, with minimal cell lysis, causes a very high rate of malignant transformation (around $1/10^3$ cells) (Fig. 9.6) [46].

SV40 produces two oncogenic proteins, Tag and tag. The large Tag is capable of inducing structural and numerical chromosomal alterations. The large Tag also induces insulin-like growth factor expression and inhibits p53 and the pRb family, and it induces c-met activity to stimulate cell proliferation. The small tag inhibits cellular phosphatase 2A, stimulates MAP kinase and AP-1 activity, and works with Tag to bind and inhibit p53 and pRb. The combined activity of both Tag and tag induce Notch-1 and telomerase activity, which are required for malignant transformation and immortalization [30].

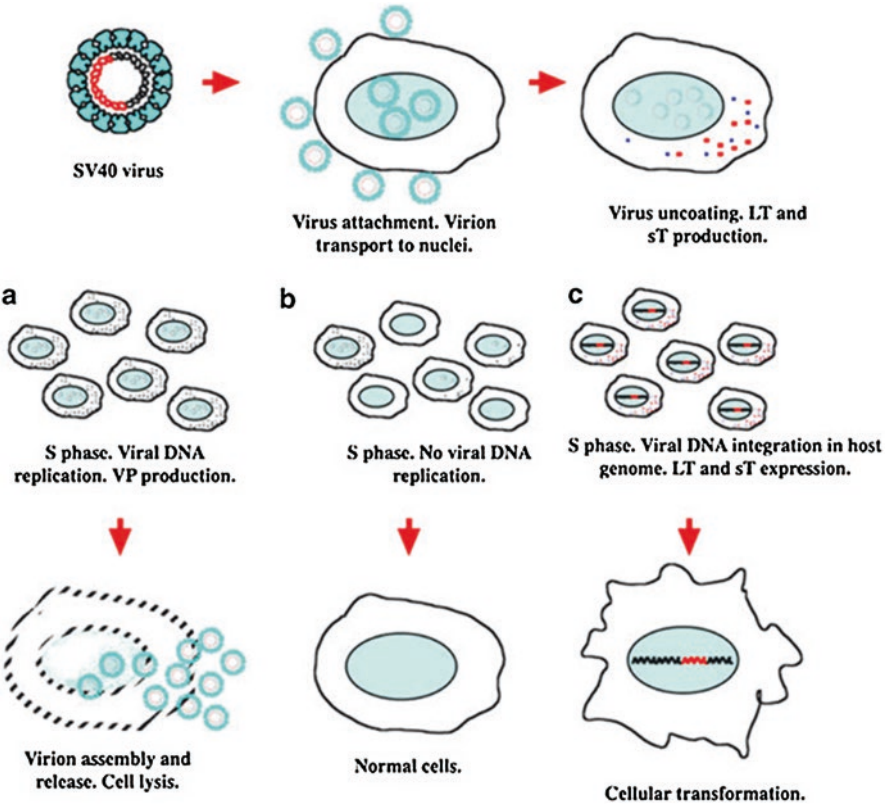

Fig. 9.5 Simian virus 40 (SV40) effects in different cellular environments. (**a**) Infection of permissive cells results in cell death and virion production. (**b**) SV40 infection of rodent cells induces S-phase but does not result in cell death or virus production. (**c**) Integration of viral DNA occurs in a very low percentage of nonpermissive cells, which then become stably transformed. *LT* large T antigen, *sT* small t antigen (Reproduced from D Ahuja et al. Oncogene 2005)

Bocchetta et al. found that p53 is not a passive inactive partner of Tag. Instead the p53-Tag complex promotes malignant cell growth through its ability to bind and activate the Insulin-like Growth Factor-I (IGF-I) signaling pathway [47]. These findings suggested that SV40 could contribute to the development of malignant mesotheliomas that occur in people not exposed to asbestos.

9.3.5 Evidence against the Roles of SV40 in Malignant Mesothelioma

Several arguments about the precise role of SV40 in the pathogenesis of all mesotheliomas remain. First, the possible impact of SV40 on overall mesothelioma incidence has not been determined. This has been limited by the fact that studies comparing

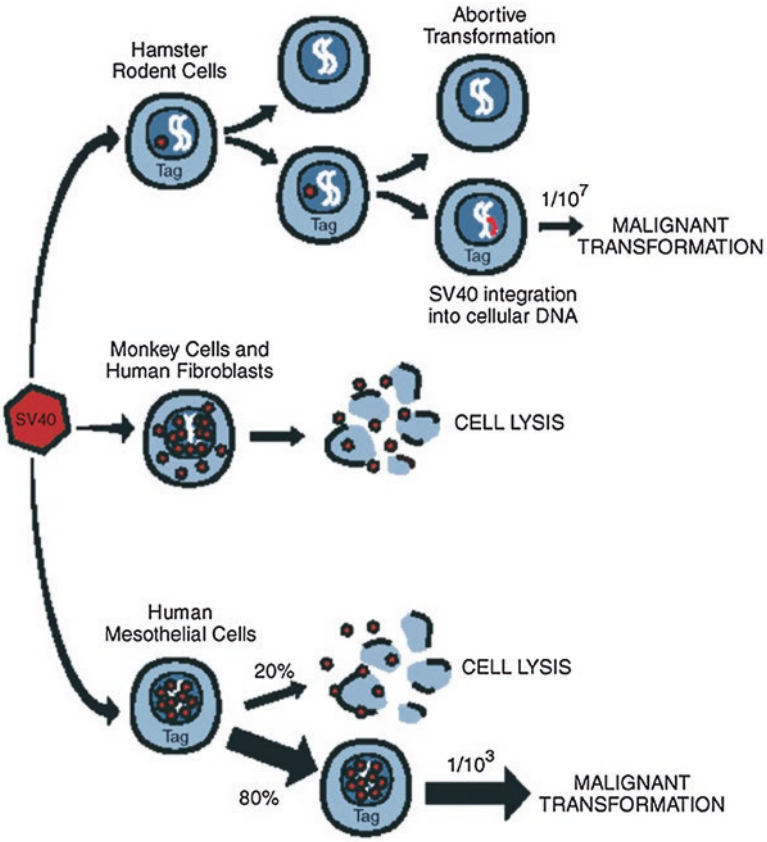

Fig. 9.6 Possible outcomes of Simian virus 40 (SV40) infection. (Top) SV40 infection of nonpermissive rodent cells, no viral particles are produced, malignant transformation is rare; (middle) SV40 infection of permissive monkey or human fibroblasts, many viral particles are produced and the cells are lysed, malignant transformation is very rare; (bottom) SV40 infection of human mesothelial cells leads to limited viral production compared to fibroblasts, limited cell lysis, and frequent malignant transformation. *Tag* large T antigen (Reproduced from M Carbone et al. Oncogene 2003)

mesothelioma incidence in SV40-infected cohorts versus non-infected cohorts are unreliable, because it seems impossible to identify infected and uninfected cohorts. Second, most mesotheliomas develop in people who have been exposed to asbestos, some of whom are SV40-negative. It may be difficult to separate the effect of SV40 and asbestos in individuals exposed to both carcinogens. Third, SV40-infected mesothelial cells should express viral antigens that would be an easy target for the immune system. Why they would not be eliminated before tumor development is unclear, but the immunosuppressive effects of asbestos may play a role. Fourth, SV40 was not found in mesotheliomas in certain countries, which indicates that, like asbestos, it is not always necessary for mesothelioma development [30].

In a retrospective study, Hirvonen et al. tested the presence of SV40-like DNA sequences in frozen tissue samples from 49 Finnish patients with MM who were not exposed to SV40-contaminated polio vaccines. They found that no SV40-specific amplification was observed in any of the mesothelioma tumor samples by PCR [48]. The results suggest that the SV40-like sequences detected in mesothelioma tissue in some previous studies may indeed originate from SV40-contaminated polio vaccines.

In another retrospective study, De Rienzo et al. found SV40 sequences in 4 of 11 mesothelioma samples from the United States but in none of the nine Turkish mesothelioma samples analyzed in the same laboratory under identical conditions using PCR [49]. The findings implied that geographical differences exist with regard to the involvement of SV40 in malignant mesothelioma.

To examine the prevalence of SV40 in malignant mesothelioma specimens in 35 patients in Japan from 1982 to 2002, Aoe et al. found that SV40 infection did not have a major role in the development of malignant mesothelioma. None of the specimens were positive with SV40 using immunohistochemical staining with anti-SV Tag antibody. Only 2 of 34 specimens were positive with SV40 using real-time PCR [50]. Reasons for low prevalence of SV40 in malignant mesothelioma in Japan are low consumption of contaminated polio vaccine in Japan (1961–1963) and ethnic difference in susceptibility to SV40, which is lower in Japanese than in other population with higher rate of SV40 infection.

By using three independent experimental approaches to detect SV40 in 71 frozen mesothelioma samples, *López-Ríos*et et al. did not support a significant role for SV40 in human mesotheliomas [51]. The first two primer sets for DNA PCR gave positive results in proportions similar to those reported in positive studies (56–62%). But these two primers in a region of the Tag gene (nucleotides 4100–4713) that is present in many common laboratory plasmids. Only 6% of specimens showed positive with less-contaminated primers. All 71 mesotheliomas were negative for Tag transcripts by real-time PCR, and lacked Tag positive tumour cells by immunohistochemistry. They suggested that inter-laboratory and geographical variations in PCR positivity for SV40 may be related less to technical factors or geographical differences in the use of SV40-contaminated polio vaccines than to the type of laboratory—i.e., whether groups carrying out the assays were in molecular-biology laboratories (with frequent plasmid work and therefore higher plasmid contamination risk) or in molecular-pathology laboratories (mostly PCR-based work with little or no plasmid work, therefore low plasmid-contamination risk) [51].

A recent retrospective study in South Korea found that SV40 is not associated with the development of malignant mesothelioma in Korea. Immunohistochemical staining demonstrated that all examined paraffin-blocks of 62 patients with malignant mesothelioma were negative for SV40 protein. Sufficient DNA was extracted for real-time PCR analysis from 36 cases. Quantitative PCR of these samples showed no increase in SV40 transcript compared to the negative controls [52].

Another argument against the evidence of supporting SV40 roles in mesothelioma development from previous reports is that the methods used to detect SV40 in those reports are not perfect. These methods include real-time PCR, sanger sequencing, pyrosequencing, and immunohistochemical staining which are used to detect

SV40 sequences or antigens in mesothelioma cells on paraffin-embedded tissues of biopsied specimens. [9, 46, 53]. They may yield false-negative results because of the low viral copy number in infected cells for molecular methods or because of the effect of formalin fixation which may result in absence of immunoreactivity for immunohistochemical staining method [54]. They may yield false-positive results because of SV40 sequences-contaminated plasmids in pathological laboratories for molecular methods [51] or because of the effects of immunostaining procedure and result interpretation for immunohistochemical staining method.

9.4 Conclusions

The mechanism of carcinogenesis in MPM is multifactorial and controversial. MPM may result from the interaction between different factors such as genetics, environmental exposure, airways microbiota and viral infection. There have been many studies supporting the close relation between SV40 and malignant mesothelioma. However, more studies are needed to confirm the potential roles of SV40 in the pathogenesis of malignant mesothelioma.

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Chapter 10
Infections Related to Development of Head and Neck Cancers

Orly M. Coblens and Jason G. Newman

Abstract Worldwide, over 550,000 new cases of head and neck cancer are diagnosed each year. Of those, approximately 119,000 are diagnosed in the United States. Head and neck cancers are predominately squamous cell carcinoma of the tongue, pharynx and larynx but they can also be other types of cancers that arise within the nasal cavity, sinuses, lips, mouth, thyroid gland, skin, salivary glands and ears. These cancers often present at an advanced stage (III or IV) and require multimodal therapy with a combination of surgery, radiation and/or chemotherapy. Alcohol and smoking are established risk factors for these cancers that increase risk independently (with tobacco exposure conveying a higher risk) and synergistically. Other important causes of head and neck cancers are infectious microbes, including but not limited to human papilloma virus (HPV), Epstein-Barr virus (EBV), and Merkel Cell Polyomavirus. The majority of this chapter will cover HPV and its implication for the development of head and neck squamous cell carcinoma (SCC), especially within the oropharynx.

Keywords Squamous cell carcinoma · Head and neck cancer · HPV · EBV · Oropharyngeal carcinoma

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10.1 Oropharyngeal Cancer

10.1.1 History of Human Papilloma Virus (HPV) and Cancers of the Head and Neck

The first study elucidating the role that HPV plays in the oral cavity was published by Syrjänen's group in 1983 [1]. It provided the seminal evidence that a subgroup (approximately 20%) of oral cancers is associated with HPV, based on detection of HPV structural proteins in these lesions using an antibody prepared against pooled HPV types (11, 16, 18) [1]. In 1989, Brandsma and Abramson [2] found that the anatomic site within the head and neck plays a role in determining the susceptibility to development of HPV related cancers. They found that SCCs of the tongue, tonsil and pharynx harbored HPV type-16 (HPV-16) related sequences in 18%, 29% and 13% of cases respectively.

Using polymerase chain reaction, Paz's group found HPV DNA in 60% (9 of 15) of patients with SCCs of Waldeyer's ring of lymphoid tissue as compared to 1 of 28 (3.6%) in the larynx, 1 of 10 (10%) in the oral cavity, 5 of 39 (12.8%) in the tongue, 2 of 15 (13.5%) in the floor of the mouth, 3 of 21 (14.3%) in the supraglottic larynx, and 1 of 7 (14.3%) in the lip. They also found a high incidence of HPV DNA within the metastatic cervical lymph nodes of those patients who had an unknown primary tumor site (3 of 8, 37.5%) [3].

In 1997 researchers were narrowing down the patient profile and found that the incidence of HPV within non-smokers was 50% versus 8.5% in smokers [4]. They also found an increased incidence of HPV within the head and neck cancers of the oropharynx (18.6%) compared to other sites. While these associations were being established it was recognized that detecting HPV DNA within tumor tissue was not sufficient evidence to claim causation; molecular proof of HPV activity would be necessary.

Further supporting evidence was also uncovered on a molecular level. This study found less retinoblastoma tumor suppressor protein (pRb), activity in 12 tonsil cancers and of those 11 (92%) also had HPV-16 DNA and a wild-type p53 protein. These were compared to nine tonsil cancers that had significant pRB activity but no detectable HPV DNA. This supports the idea that HPV-16 may function in oral carcinogenesis through E7-mediated inactivation of pRb [5]. Mork et al., performed a case-control study evaluating serum antibodies to viral capsid proteins for HPV types 16, 18, 31, and 73 that were collected approximately 10 years prior in a Nordic population and found that those with serological evidence of HPV-16 infection had a 14-fold increase in the risk of developing oropharyngeal cancer compared to those who were serologically negative. The overall odds ratio for SCC of the head and neck in subjects who were seropositive for HPV-16 was 2.2 (95% confidence interval, 1.4–3.4). This proved a temporal relationship between exposure and risk for head and neck SCC [6].

A few years later, using a similar method of serum evaluation, Smith et al. confirmed that individuals with seropositive HPV-16 E6 and E7 antibodies had 73 times

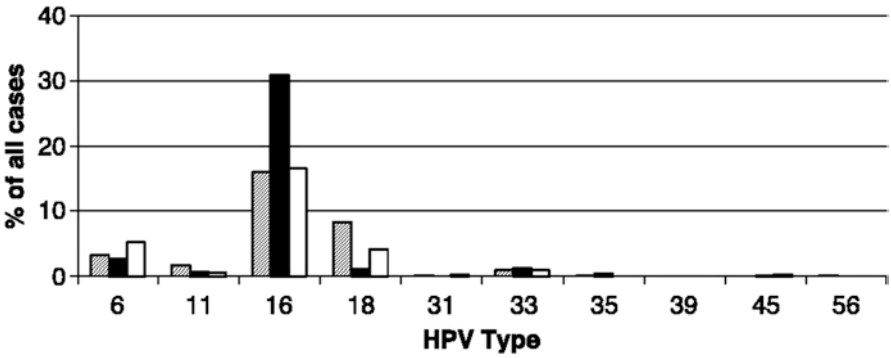


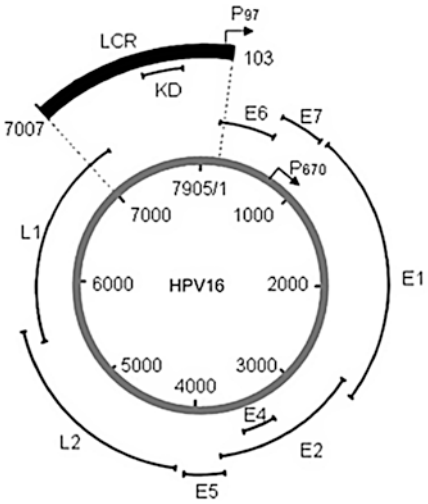
Fig. 10.1 Type-specific prevalence of in full HPV in 2642 oral cavity in full SCCs, 969 oropharyngeal SCCs, and 1435 laryngeal SCCs. Columns with diagonal lines, oral SCCs; black columns, oropharynx SCCs; white columns, laryngeal SCCs. Larynx includes SCCs of the hypopharynx [8]

the risk of developing oropharyngeal cancer compared to those who were seronegative (OR, 72.8; CI 95%, 16.0–330) [7].

Kreimer et al. published a systematic review in 2005 detailing the HPV site and subtype within the published literature of head and neck cancers. They found that HPV prevalence was significantly higher in oropharyngeal SCCs (35.6% of 969; 95% CI, 32.6–38.7) than oral SCCs (23.5% of 2642; 95% CI, 21.9–25.1) or laryngeal SCCs (24.0% of 1435; 95% CI, 21.8–26.3). HPV-16 accounted for a larger majority of HPV-positive oropharyngeal SCCs (86.7%; 95% CI, 82.6–90.1) compared with HPV-positive oral SCCs (68.2%; 95% CI, 64.4–71.9) and laryngeal SCCs (69.2%; 95% CI, 64.0–74.0). They concluded that the HPV-related cancers of the head and neck most commonly affected the oropharyngeal tonsillar tissues and around 90% of those were caused by a single HPV-16, followed by HPV type-18 (HPV-18) (Fig. 10.1) [8].

10.1.2 How Does HPV Cause Oropharyngeal Cancer?

Human papilloma viruses are double-stranded circular DNA viruses with an icosahedral capsid from the *Papillomaviridae* family that have a predilection for infecting mucosal or cutaneous squamous epithelia. There are more than 100 subtypes of HPV, however only a few have been determined to have oncogenic potential and those are referred to as the high-risk types. These include but are not limited to types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, and 59 [9]. HPV-16 was discovered in the 1970s and its role as an oncogenic virus has been determined and especially well categorized within the framework of cervical cancer [10] (see Chap. 13).



Viral Protein	Functions and Features
E1	It forms a heterodimer complex with E2 and controls viral replication
E2	It regulates early gene promoter and together with E1 viral DNA replication
E4	It may mediate the viral particle realease by destabilizing cytokeratin network
E5	It stimulates mitogenic signals of growth factors
E6	It inactivates many cellular proteins and is one of the major viral oncoproteins
E7	It inactivates many cellular proteins and is one of the major viral oncoproteins
L1	It is the major capsid protein and is the component of the HPV prophylactic vaccine
L2	It is the minor capsid protein

Fig. 10.2 The double-stranded DNA HPV16 genome is represented by a grey circle annotated with the nucleotide numbers. The positions of the long control region (LCR) and the early genes (E1–7) and late genes (L1 and L2) are also shown. The early and late promoters, P97 and P670, respectively, are indicated by arrows. The main functions and features of the early and late gene products are listed in the table [11]

The HPV genome is around 8000 base pairs and can be divided into three different regions (Fig. 10.2):

1. Early genes: code for proteins that regulate viral DNA replication, E1, E2, E4, E5, E6, and E7.
2. Late genes: code for the capsid proteins, major (L1) and minor (L2).
3. Long control region (LCR): a non-coding region, localized between open reading frame (ORFs) L1 and E6 and contains most of the regulatory elements involved in viral DNA replication and transcription.

The early genes, E6 and E7, contain the main oncogenes whose expression inactivates p53 and pRB respectively. This causes a disruption in the cell regulators and is considered to be the onset of HPV-mediated carcinogenesis.

HPV infects epithelial cells. These cells, which are organized in layers, cover the inside and outside surfaces of the body, including the skin, upper aerodigestive tract, genital tract, and anus. The HPV infection occurs via introduction of the virus to the basal layer of epithelial cells. The mucosal lining of the palatine and lingual tonsils within the oropharynx is unique in its close relationship to the lymphoid tissue of Waldeyer's ring which is the first line of defense for the aerodigestive tract. The tonsillar epithelium's surface area is maximized by the architecture of the tonsillar tissue with blind crypts that extend the full thickness of the tonsil (Fig. 10.3). The tonsillar crypts are lined by reticulated epithelium that results in an incomplete basement membrane enabling the passage of lymphocytes and antigen-presenting

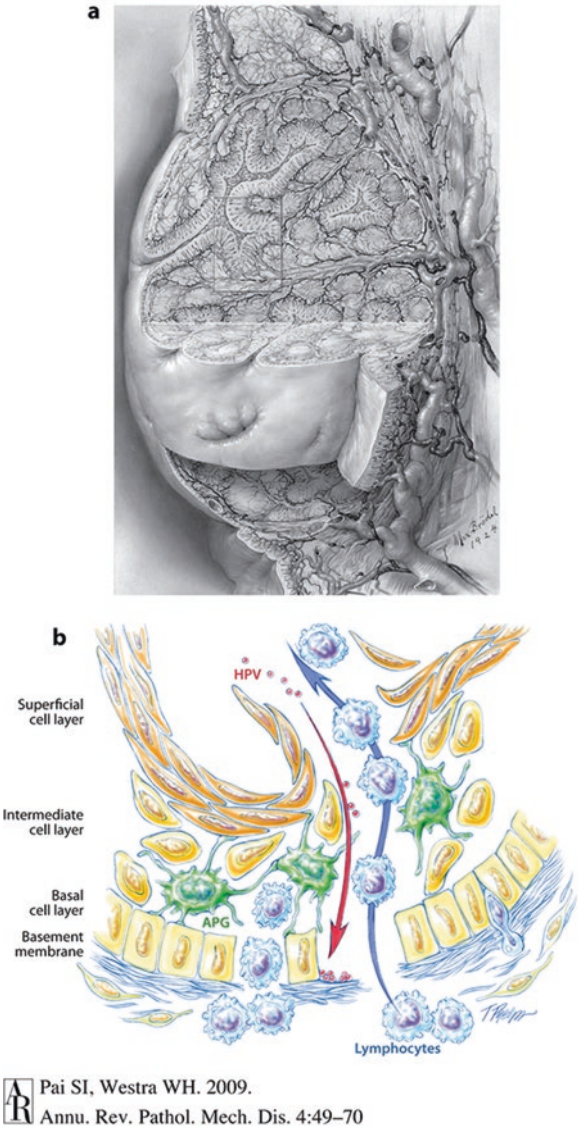


Fig. 10.3 (**a**) Topography of the human palatine tonsil. The surface epithelium of the palatine tonsil deeply invaginates into a lymphoid stroma as blind-ending and ramifying crypts (boxed area) that increase the surface area of the tonsil by nearly 700%. Drawing by Max Brödel. Used with permission from Art as Applied to Medicine, the Johns Hopkins University School of Medicine. (**b**) The specialized reticulated epithelium lining the tonsillar crypts. The zones of squamous epithelium—the basal, intermediate, and superficial layers—are interrupted by migrating nonepithelial cells including lymphocytes and antigen-presenting cells. Loss of structural integrity leaves the basement membrane exposed to deposition of viral particles. Drawing by T. Phelps. *APG* antigen presenting group, *HPV* human papillomavirus [12]

cells [13]. In 2005, Begum et al. compared neoplastic tonsillar tissue with contralateral non-neoplastic tissue and found there was no evidence of "field cancerization" with regard to HPV-16 DNA integration. They did find that HPV-16 DNA was present in dysplastic and metastatic tissues and that the cells with integrated HPV DNA were present in the reticulated epithelial lining of the crypts [14].

Once the virus integrates its DNA genome into the host cell nucleus, it dysregulates expression of the oncoproteins E6 and E7. The E6 protein induces degradation of P53 through ubiquitin-mediated proteolysis, leading to loss of P53 activity. The usual function of P53 is to arrest cells in G1 or induce apoptosis to allow host DNA to be repaired. E6-expressing cells are not capable of this P53-mediated response to DNA damage and, hence, are susceptible to genomic instability. The E7 protein binds and inactivates pRb, causing the cell to enter S-phase, leading to cell-cycle disruption, proliferation, and malignant transformation [15]. Furthermore, the cell cycle components Cyclin D1 and p16INK4a which are regulated by pRb, are also affected with reduced expression of Cyclin D1 and overexpression of $p16^{INK4A}$ [5]. The upregulation of $p16^{INK4A}$ reaches levels that are detectable by immunohistochemistry. This staining is 100% sensitive but only 79% specific as a surrogate marker for an HPV mediated carcinoma [16].

10.1.3 Clinical Presentation

The oropharynx is made up of the palatine tonsils and tonsillar pillars, soft palate and pharyngeal walls as well as the lingual tonsils and the base of tongue. It is important for respiration, deglutition, production of speech and taste. Patients that have any difficulty with these tasks can present for evaluation. Historically, the majority of the head and neck cancer patients have been older with a strong history of cigarette smoking and alcohol use who presented with cancers throughout the upper aerodigestive tract. Over the past three decades there has been a significant decrease in this group of patients but an increase in patients with primarily oropharyngeal cancer that is driven by HPV [17]. The percentage of HPV-positive oropharyngeal SCC increased from 16.3% in 1984–1989 to 70% in 2000–2004 in the United States based on Surveillance, Epidemiology, and End Results Program (SEER) database. Similarly in Sweden, the Netherlands and the United Kingdom there has been a growth of OPSCC despite decreases at other sites [18–21]. HPV-positive oropharyngeal SCC is different from conventional HNSCC in its clinicopathologic features and molecular pathogenesis (Table 10.1).

The patients presenting with HPV-positive oropharyngeal SCC tend to be younger, predominately male with minimal alcohol and tobacco exposure, they are associated with certain high-risk sexual practices such as a high-lifetime number of vaginal-sex partners and a high-lifetime number of oral-sex partners, and higher socioeconomic status [22, 23]. While exposure and viral detection is common, 6.9% in those aged 14–69 years of age, the conversion into malignancy is not and there-

Table 10.1 Differences in the clinical and biologic features between HPV-negative and HPV-positive head and neck SCC

	HPV-positive head and neck SCC	HPV-negative head and neck SCC
Risk factors	High-risk sexual practices	Cigarette smoking and alcohol use
Primary tumor site	Oropharynx—palatine and lingual tonsils	No predilection
Histopathology	Basaloid, non-keratinizing, poorly differentiated	Keratinizing, moderately differentiated
Concurrent cervical lymph node involvement	Significant	
Incidence	Increasing	Decreasing
Age at time of diagnosis	Under 60	Over 60
Molecular genetic alterations		
p53 pathway disturbances	Degradation of wt p53 by E6	TP53 mutations, 17p LOH
pRB pathway disturbances	Degradation of wt Rb by E7	$p16^{INK4A}$-promoter hypermethylation, 9p LOH
P16 protein	overexpressed	No significant change
Relative responsiveness to chemoradiation	Better	Worse
Relative prognosis	Improved	Worse

Adapted from Pai and Westra [12]

fore we assess pathologically for the overexpression of p16 as a surrogate marker for the oncogenic conversion [24].

Finally, HPV-positive oropharyngeal SCC has a distinct histological appearance; it is poorly differentiated and non-keratinizing with an associated basaloid morphology and positive p16 immunohistochemistry [25] (Fig. 10.4).

10.1.4 Treatment and Prognosis

For patients with HPV-positive oropharyngeal SCC, there is an improved overall survival and disease-free survival compared to patients with HPV-negative tumors [25, 27–29]. Eastern Cooperative Oncology Group (ECOG) 2399 prospectively found that patients with HPV-positive oropharyngeal SCC treated with induction chemotherapy followed by definitive chemoradiotherapy had higher response rates after induction chemotherapy and after definitive chemoradiotherapy as compared to HPV-negative oropharyngeal SCCs. They also found that overall survival was improved [30]. Ang et al. specifically found that patients with advanced (stage III or IV) HPV-positive oropharyngeal SCC treated with chemotherapy and radiation had a 3-year survival rate of 82.4% compared to 57.1% for those patients with

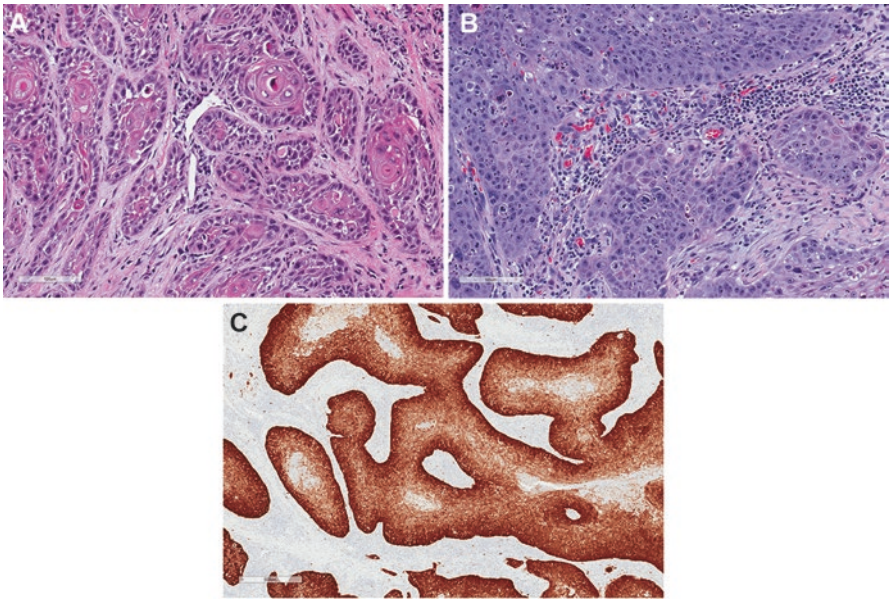


Fig. 10.4 The histology of conventional and HPV-associated head and neck squamous cell carcinomas (HNSCC). (**A**) Well differentiated conventional type HNSCC with keratinization (hematoxylin-eosin, 200×). (**B**) HPV-associated HNSCC which lacks keratin and has a poorly differentiated basaloid histology (hematoxylin-eosin, 200×). (**C**) The same tumor in part **B** with diffuse and strong nuclear and cytoplasmic p16 staining (p16 immunohistochemistry, 40×). (Images Courtesy of Suimen Qiu, MD) [26]

HPV-negative oropharyngeal SCC. However, the risk of death was significantly increased with each additional pack-year of tobacco smoking [31].

The overall improved therapeutic response to radiation by HPV-positive tumors may result from its carcinogenic mechanism [32]. These tumor tissues have a greater intrinsic radiation sensitivity, and with an intact p53 protein (even at low levels), the apoptotic response remains intact as well. Additionally, radiation induces changes of tumor surface protein expression enabling greater participation by the host immune system to assist in clearance [33].

The diagnostic, prognostic and non-surgical management of HPV-positive oropharyngeal SCC has been advancing over the past three decades, along with surgical technology which remains to have a major impact on the overall management, treatment and survival for these patients. Transoral approaches to the oropharynx using laser microsurgery or transoral robotic surgery (TORS) have proven to be safe and effective for locoregional control with improved quality of life outcomes [34]. TORS has been utilized as a means to decrease the intensity of radiotherapy and avoid chemotherapy in 38–80% of patients even with advanced stage disease.

Importantly, it has been found to decrease feeding tube dependence [35]. Based on the evidence for surgical success and improved quality of life, a randomized multicenter control trial was established, ECOG 3311. This study's main objective is to investigate the utilization of index surgical resection with de-intensified adjuvant therapy (comparing 50 Gy vs 60 Gy) for patients with intermediate-risk HPV-positive oropharyngeal SCC.

10.1.5 Future Directions

In the United States there are currently three FDA-approved vaccines available against HPV: a bivalent HPV-16/18 vaccine (Cervarix®, GlaxoSmithKline Biologicals), a quadrivalent HPV-6/11/16/18 vaccine (Gardasil™, Merck Sharp and Dohme) and a nanovalent HPV-6/11/16/18/31/33/45/52/58 (Gardasil™). With reference to cervical cancer, prospective clinical trials have demonstrated that premalignant lesions can be prevented by HPV vaccination and detected by screening for HPV infection. Given the success of these vaccines in cervical cancer prevention, it is postulated that vaccination may be similarly successful in preventing head and neck cancer. A double-blinded study by Herrero found 93.3% vaccine efficacy against oral infections with HPV-16/18 in women in Costa Rica 4 years after receiving vaccination [36]. Another study demonstrated that vaccination with the quadrivalent vaccine induced HPV antibodies in the oral cavity of males that correlated with the level of circulating antibodies [37]. Using the National Health and Nutrition Examination Survey (NHANES), vaccinated adults (age 18–30 years) were found to have a lower prevalence of HPV 6, 11, 16, 18 compared to unvaccinated adults [38]. Gillison has demonstrated that the prevalence of oral HPV-16/18/6/11 was significantly reduced in vaccinated versus unvaccinated individuals (0.11% vs 1.61%) [39]. There have also been trials in the United Kingdom and the United States that have utilized vaccine immunity, delivering HPV-16 E7 antigen as adjuvant therapy with efforts to augment the T-cell mediated immune response for patients with HPV-positive oropharyngeal SCC. It has yet to be determined whether HPV vaccination and decreased oral HPV infections will prevent the development of oropharyngeal SCC or other head and neck SCC.

10.2 Nasopharyngeal Cancer

Nasopharyngeal carcinoma (NPC) is a rare head and neck cancer outside of southern Asia. Many factors play a role in the development of this cancer including infections with EBV, consumption of nitrosamines in pickled foods or salted fish, and smoking tobacco [40]. At least 95% of NPCs are associated with EBV. It has been recognized that testing for the presence of EBV DNA in plasma samples is 97.1% sensitive and 98.6% specific in identifying early asymptomatic NPC [41]. Further

studies are required to understand the contributions of EBV to the initiation and development of NPC and the differences that occur in different geographic locations.

10.3 Laryngeal Cancer

The larynx (voice box) is made up of the supraglottis, glottis and subglottis and it is primarily responsible for respiration, phonation, and airway protection. In the United States in 2017 there were an estimated 13,360 new laryngeal cancers diagnosed and 3660 deaths from laryngeal cancer [42]. Since the early 1990s and the publication of the RTOG 91–11 studies [43, 44] the mainstay of treatment has been organ preservation with chemoradiotherapy and salvage total laryngectomy. Despite the advances in cancer treatment and reduction in tobacco-related cancers, specifically laryngeal cancer, there is still a declining overall 5-year survival rate [42, 45].

The amount of tobacco and alcohol use have a linear association with the development of laryngeal cancer [46, 47]. The carcinogenic role of other environmental factors is important [48, 49], but the role that HPV plays in laryngeal carcinogenesis is still being determined.

It was first postulated in 1978 that benign laryngeal papillomas were caused by exposure to HPV via genital condylomatous lesions [50]. These viruses replicate in the multi-layered squamous cell epithelium and develop into a papilloma that is usually benign but can cause changes in voice and respiration. Subsequently it was discovered that HPV-types 6 and 11 were associated with recurrent respiratory papillomatosis (RRP) within the aerodigestive tract [51]. Compared to papillomas caused by HPV-6, those caused by HPV-11 are associated with more aggressive disease [52] and risk of malignant transformation [53]. Jeong notes that malignant transformation of these papillomas occurs in 1–4% of patients with RRP. He also notes that of the 44 published cases within the literature, 25 were associated with HPV-11, four with HPV-6 only, and another six with low-risk HPV. Five specimens had high-risk HPV types (HPV-16 or − 18) [54].

In patients with verrucous carcinoma of the larynx, 40–85% have detectable HPV DNA. However, no prognostic significance has yet been found [55–57].

Finally, while high-risk HPV DNA has been detected in laryngeal SCC, there have been no case-control studies or large enough studies with standardized identification techniques to provide etiological proof that HPV DNA plays a significant role in laryngeal carcinogenesis [58]. With various detection techniques, the prevalence of HPV is around 25% in laryngeal SCCs [8, 59]. Additionally, the largest laryngeal SCC series comparing HPV-positive and HPV-negative tumors showed no significant difference in overall and disease-specific survival at 3 and 5 years [60].

10.4 Oral Cavity Cancer

The oral cavity is comprised of the lips, alveolar ridge, buccal mucosa, retromolar trigone, floor of mouth and oral tongue. It is the most common site for SCC in the upper aerodigestive tract. The most significant risk factors for development of cancers of the oral cavity are tobacco and alcohol use but also include smokeless tobacco and betel quid use. Within the oral cavity, the mobile tongue is the most common site for SCC. Despite the decreasing trends of smoking and alcohol use there has been an increase in a subset of oral tongue SCCs amongst young non-smokers and non-drinkers [61]. The reason for this increase has yet to be determined however a potential infectious etiology is plausible.

10.4.1 Chronic Infection/Periodontal Disease

Periodontal disease, which includes gingivitis and periodontitis, is highly prevalent in adults and disease severity increases with age. Gingivitis is inflammation of the gums and is considered early periodontal disease that is reversible. Periodontitis occurs via the accumulation of dental plaque, bacterial overgrowth, formation of periodontal pockets, gum recession, tissue destruction and alveolar bone loss. In the United States, national health surveys have reported a high prevalence of periodontitis on the basis of oral health examinations; the prevalence of periodontitis in dentate adults over the age of 30 years is estimated to be around 47% and increases to 70% in individuals 65 years of age or older [62].

The relationship between periodontal disease and systemic health has been investigated for many years and associations have been found with obesity, respiratory conditions like COPD, cardiovascular disease, diabetes, and arthritis [63]. More recently researchers have found a link between periodontal disease and overall (non-head and neck) cancer risk, with systemic inflammation serving as the main hypothesis for biological likelihood [64].

In a case-control study each millimeter of alveolar bone loss was associated with a greater than fourfold increased risk of head and neck SCC (OR 4.36; 95% CI 3.16–6.01) after adjustment for age, gender, race/ethnicity, marital status, smoking status, alcohol use, and missing teeth. The strongest association was in the oral cavity followed by the oropharynx and then the larynx [65]. They discovered that each millimeter of bone loss was associated with a 5.23-fold increase risk of specifically tongue cancer [66]. A meta-analysis also found a significant association of periodontal disease with an increased susceptibility to oral cancer (OR 3.53; 95% CI 1.52–8.23) [67]. Finally, a recent systematic review of nine studies reported a two- to five-fold increased risk of oral cavity cancer in patients with periodontal disease compared to those without. These associations were also found to be attenuated after adjusting for tobacco and alcohol use [68].

Overall, periodontal disease is associated with increased risk for oral cavity SCC; however, a causal relationship has yet to be determined.

With recent advances in high-throughput sequencing, investigative efforts have been focused on the role that the oral and salivary microbiome has on the development of oral cavity cancer. Through the production of toxins, chronic inflammation and carcinogenic products, bacteria have played a role in human carcinogenesis. The oral cavity contains hundreds of species of bacteria, viruses, fungi, archaea and protozoa [69]. Many studies have demonstrated a difference between the oral microbiome of individuals with and without oral cavity SCC [70]; however, the role that this shift in microbial entities has on the development of cancer and the interplay between systemic exposures has yet to be elucidated. Use of this advanced diagnostic technology may enable the detection of risk factors to help prevent oral cavity cancers in the future.

10.4.2 HPV

The first publication highlighting the role of HPV in head and neck SCC found that 20% of oral cancers were associated with HPV, based on detection of HPV structural proteins [1]. While oral infections with HPV are present in 6.9% of the population (1.0% are HPV-16) [24], its carcinogenic effects are still under investigation. Several studies have investigated prevalence of HPV DNA in these cancers, but the detection methods vary, depending on the population, combination of subsites (contamination by tumors that are actually oropharyngeal primaries), types of specimens, and confounding variables. In a systematic review of 60 studies comparing oropharyngeal, oral cavity and laryngeal SCCs 25.9% HPV prevalence overall was found. The HPV prevalence in oral cavity SCC was 23.5% of the 2642 cases worldwide. HPV-16 and HPV-18 were found in 68.2% and 17% of the positive cases respectively [8]. They also found the HPV prevalence from oral cavity SCC was higher in Asia. The International Agency for Research on Cancer study found HPV DNA in only 3.9% of oral cavity SCCs [36]. Another large study showed that 16.8% of 4195 oral cavity SCC tumor specimens contained HPV DNA [71].

Since the previous studies utilized HPV DNA detection via PCR, which is very sensitive but not specific, and did not investigate molecular markers of HPV oncogenic activity, causality cannot be concluded. Some studies have assessed the molecular markers of E6/E7 mRNA as well as p16 protein overexpression and found very limited support for HPV carcinogenesis within the oral cavity despite DNA detection [72].

Overall, determining the role that HPV plays in the development of oral cavity SCC is problematic because of many confounding variables that still play a significant role (tobacco smoking and alcohol use) and methodology which has not consistently evaluated biomarkers of oral cavity HPV carcinogenesis.

10.4.3 EBV

The role that EBV plays in NPC has been well established however its role within the development of oral cavity SCC is debatable. One study evaluated 98 patients with mobile tongue SCC using three different methods of detection and did not find an EBV association [73]. This, however, was done on tissue bank samples and may not represent the current patient population. In contrast, a meta-analysis of 13 case-control studies found that EBV infection does increase the risk of oral cavity SCC [74]. This study did show heterogeneity; therefore, studies with a larger sample size maybe helpful in determining the carcinogenic role that EBV plays, if any, within this subsite of the head and neck.

10.5 Head and Neck Skin Cancer

Cutaneous malignancies are the most common malignancy in the United States; however, current cancer registries do not account for these tumors and therefore studies about them are based on large institutional studies or insurance/insurance records. A majority of cutaneous malignancies, especially basal cell carcinoma and SCC, are found in the head and neck. Skin cancer and the role that microbiomes play is important. Please refer to Chap. 4 for more details.

10.5.1 Merkel Cell Carcinoma (MCC)

The most common site for Merkel cell carcinoma in the United States is the face/neck/scalp (48%), followed by the upper limb (19.3%), lower limb (16.0%), trunk (11.3%) and then other sites (5.2%) [75]. Its association with Merkel cell polyomavirus is significant and may provide insight into treatment options that can help improve the survival outcomes. Please refer to Chap. 11 for more details.

10.6 Sinonasal Cancer

The sinonasal cavity consists of the nasal cavity, including the olfactory region, and the paranasal sinuses (maxillary, ethmoid, sphenoid and frontal sinuses). Primary sinonasal cancer represents less than 3% of all head and neck cancers. The most common malignancies are SCC (51.6%) and adenocarcinoma (12.6%), whereas the most common primary sites are the nasal cavity (43.9%) and maxillary sinus (35.9%) [76].

In a study of 161 sinonasal carcinomas, 34 (21%) were positive for high-risk HPV DNA, including type 16 (82%), type 31/33 (12%), and type 18 (6%). Of the carcinomas assessed, the SCCs were most likely to be HPV-related. The HPV-positive tumors had high p16 expression in 33 of 34 (97%) of cases, which was significant compared to the HPV-negative tumors where only 26 of 127 (20%) were p16 positive [77]. Approximately 25% of sinonasal SCC is associated with high-risk HPV, and this cohort may have improved outcomes but the overall prognosis for this diagnosis remains poor and the exact role that HPV plays is yet to be determined [78].

Finally, inverted papilloma (IP) is a benign tumor, but it is locally aggressive in the sinonasal region where it represents only a small percentage of all sinonasal neoplasms. These tumors can grow to be bulky and often produce nasal obstruction. An association with SCC has been reported in 7% of cases [79]. Because of its papillomatous histological appearance, several studies have attempted to identify a relationship between HPV, IP, and subsequent malignant transformation. Most studies looking at the presence of HPV DNA and cell cycle regulation markers have produced conflicting evidence [80]. Another study investigated the role that EBV plays in IP and found that 65% of specimens in a case-control series were positive for EBV DNA; however, this was not associated with increased incidence of cancer in the sinonasal cavity [81].

10.7 Salivary Gland Cancer

The salivary glands of the head and neck include the parotid, submandibular, sublingual and minor salivary glands. The common types of salivary gland cancer are adenoid cystic, myoepithelial, mucoepidermoid, acinic cell, epithelial-myoepithelial, adenocarcinoma, and SCC. Salivary gland malignancies represent 5% of all head and neck cancers.

Undifferentiated carcinoma of the salivary glands has a poor prognosis and is histologically indistinguishable from lymphoepithelial undifferentiated carcinoma of the nasopharynx. Many researchers have found that these undifferentiated carcinomas with lymphoid stroma of the salivary glands also contain EBV genetic material [82–84]. EBV has also been found in varying degrees (0–95%) in benign pathologies including pleomorphic adenoma and Warthin's tumors [85–87]. Overall, its role as a causative agent is debatable [88].

Through various techniques HPV DNA has been detected in benign and malignant parotid tumors. Interestingly, 47.2% of patients with mucoepidermoid carcinoma had high-risk HPV E6/E7 RNA and a subset of these also had E6 protein demonstrated via immunofluorescence. This suggests a potential role for HPV-16 or HPV-18 in carcinogenesis of these tumors [71]. In a recent multi-institutional study the prevalence of 62 DNA viruses was assessed in 100 salivary gland specimens. Of the samples, 28 were normal salivary tissue, 79 were benign salivary tumors and five were malignant tumors. They found polyomavirus DNA in normal and neoplas-

tic glands. EBV1 DNA was prevalent in Warthin's tumors and beta-HPV may be associated with malignancy [89].

Overall, because these tumors are rare and the present literature has utilized various detection strategies, definitive conclusions about the role that infectious agents play in carcinogenesis of salivary gland malignancies cannot be drawn, but there is enough information to warrant further investigation.

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Chapter 11
The Microbiota and Ovarian Cancer

Janos Tanyi and Andrea Facciabene

Abstract The cellular components of the immune system and the inflammatory milieu that it can generate is a central theme in many diseases including cancer. Immune cells can be manipulated by tumor cells to favor a pro-tumor microenvironment resulting in tumor progression. Ovarian cancer can alter its microenvironment favoring tumor growth by suppressing effector T cells as well as recruiting myeloid-derived cells, Th17 cells, γδ T cells, as well as non-immune cells such as adipose cells to aid in the generation or the propagation of the pro-inflammatory milieu. The human microbiome maintains a delicate balance between pro- and anti-inflammatory mechanisms, essential for gut homeostasis, and has critical roles in immune system development and metabolism. Alterations in the microbiome results in dysbiosis, quantitative and qualitative shifts in microbial populations, and contributes to chronic inflammation in various diseases including cancer. We highlight the role that the gut microbiota may play in cancer initiation and/or progression as well as its impact on cancer therapy. The association and interactions between the microbiome, both gut microbiota as well as infectious virus, with ovarian cancer, is reviewed here. Understanding the mechanisms by which the microbiome modulates the innate and adaptive immune response and contributes to an inflammatory milieu in cancer may offer insights into novel therapeutic targets.

Keywords Gut microbiota · Microbiome · Dysbiosis · Inflammation · Ovarian cancer

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Abbreviations

HBV	*Hepatitis B* virus
HCV	*Hepatitis C* virus
HHV	Human herpesvirus
HPV	Human papillomavirus
HTLV-1	Human T-lymphotropic virus type *1*
KSHV	Kaposi's sarcoma-associated herpesvirus
LCMV	Lymphocytic choriomeningitis virus
MMTV	*Mouse mammary tumor virus*

11.1 Inflammation and Ovarian Cancer

11.1.1 Ovarian Cancer Introduction

Ovarian cancer is the most lethal gynecological cancer [1]. Ovarian cancer is a heterogeneous disease with several different histological types (serous: low and high grade, clear cell, endometrioid and mucinous) [2, 3]. Involvement of peritoneal structures contributes to poor overall survival for the most common ovarian cancer: epithelial ovarian carcinoma (EOC) [4]. EOC has a high mortality due to few specific symptoms at initial stages, leading to delayed diagnosis and treatment. High grade serous ovarian cancer (HGSC), the most lethal and frequent type of EOC, has poor long term prognosis due to a combination of factors: late detection, high metastatic potential and the capacity to develop resistance to available therapeutic drugs. HGSC likely originates not from the surface of the ovary, but from the epithelial layer of the neighboring fallopian tube fimbriae [5, 6] and consequently, removing of fallopian tubes (salpingectomy) is emerging as a prophylactic option in addition to ovary removal (oophorectomy) [7]. Since serous peritoneal, fallopian tube, and ovarian cancers are histologically and morphologically similar regardless of where they begin, and are treated alike, they have been collectively categorized as ovarian cancer [8]. Methods for screening and early diagnosis of ovarian cancer in asymptomatic women have been largely ineffective [9]. Screening and prevention is limited by the lack of sensitive and specific biomarkers which can be used to detect early malignancy. CA125 is expressed in most high-grade serous carcinoma, but only in 60% of mucinous and clear cell subtypes [10]. Some physicians use CA125 monitoring and endovaginal ultrasound for high risk patients, prospective validation remains elusive however since CA125 is neither specific nor sensitive [8, 11, 12]. In 2008, HE4 was approved for use in monitoring patients with a known diagnosis of ovarian cancer, able to detect recurrence of epithelial cancers 2–3 months in advance of CA125. Like CA125, HE4 does not have a preoperative diagnostic indication. In 2009, the first preoperative serum biomarker test for ovarian cancer was approved, a 5-protein panel called Ova1, the first multivariate index assay (MIA) [8]. Ova1 combines the second generation CA125-II with transferrin, β2 microglobulin,

apolipoprotein A1 and transthyretin into a test result of low or high risk for ovarian cancer. CA125 and HE4 (Risk of Ovarian Malignancy Algorithm, ROMA) was also approved for preoperative testing. These MIA tests significantly improved preoperative testing compared to single biomarker tests because of increased sensitivity but are not true diagnostic tests, but rather triage or referral tests. When surgery is considered for ovarian cancer, these tests are used to determine the likelihood of malignancy. A primary care provider can utilize the test to determine whether referral to a gynecologic oncologist is indicated [8]. Although biomarkers have made progress in the preoperative setting, there is still a lack of diagnostic biomarkers in early disease. Additionally, there are no ovarian carcinoma tissue-based prognostic markers used clinically, despite many candidates, because prognostic effects have proven difficult to validate and support the view that ovarian carcinoma subtypes are different diseases [2].

11.1.2 Links Between Inflammation and Cancer

Inflammation is an essential two-pronged beneficial response of the immune response in an attempt to defend itself against invasion from foreign invaders (specifically infection with bacteria, viruses and fungi) as well as heal the body after injury by repairing damaged tissue. Acute inflammation due to infection is elicited and usually resolved quickly by using pattern-recognition receptors (PRR) such as TLRs to sense and respond to injured cells and heal tissue [13, 14]. The innate immune response senses infection via PRR and is the rapid response to resolve the infection with minimal damage to cells and tissues. In contrast, adaptive responses take time to develop and are T cell- and B cell-based responses for long term surveillance. Innate immune responses can modulate adaptive immune responses via myeloid-derived (bone marrow) cells such as dendritic cells (DCs), antigen presenting cells (APC) that have specialized functions depending on their location in the body, or other myeloid-derived cells [15, 16]. Multiple mechanisms for induction of an immune response exist with the general principle that innate cells expressing PRR detect viral or bacterial antigens which elicits a first set of pro-inflammatory cytokines which in turn activate different subsets of lymphocytes (adaptive response) to produce a second set of cytokines that activates effector responses such as cytotoxic T lymphocyte (CTL) responses [15].

The initial association between inflammation and cancer has historically been attributed to Virchow, based on detection of inflammatory infiltrates in solid malignancies, and has since gained strong epidemiological and mechanistic support [14, 17]. A role for inflammation in cancer initiation and cancer progression is now generally accepted, and an inflammatory microenvironment is a component of many cancers [18]. The development of cancer from preceding inflammatory lesions is well established, including gastritis leading to gastric cancer [19], pancreatitis leading to pancreatic cancer [20], hepatitis leading to liver cancer [21] as well as intestinal bowel disease (IBD) leading to colon cancer [22]. Inflammation is a key

hallmark of cancer [18, 23, 24], a well-established tumor promoter that contributes to cancer growth, angiogenesis, and resistance to apoptosis or cell death [17, 21]. Key features of cancer-related inflammation include the infiltration of white blood cells, tumor-associated macrophages (TAMs), the presence of cytokines such as tumor necrosis factor (TNFα), interleukin (IL)-1, IL-6, chemokines such as CCL2 and CXCL8 and the occurrence of tissue remodeling and angiogenesis [25].

11.1.3 Links Between Inflammation and Ovarian Cancer

The pathophysiology underlying epithelial ovarian cancer is not clearly established [26]. Historically prevailing hypotheses include the ovulation hypothesis which relates ovarian cancer risk to incessant ovulation and the pituitary gonadotropin hypothesis, which implicates elevation in gonadotropin levels acting in concert with estrogen [27]. The ovulation hypothesis states that excessive ovulation damages the ovarian and fallopian fimbriae epithelium, from which epithelial ovarian cancer arises due to enhanced potential for aberrant DNA repair, inactivation of tumor-suppressor genes, and subsequent mutagenesis [27]. Monthly ovulation is considered to be a major event triggering inflammatory signaling at regular intervals in both the ovary and the adjacent fallopian fimbriae [5]. Parity as well as prolonged lack of ovulation for a year or more, is known to reduce ovarian cancer risk by 29%, with each new pregnancy further reducing the rate by 8%. In contrast, late menopause, associated with ovulation for a longer time period is associated with a significantly higher risk for ovarian cancer [5]. The pituitary gonadotropin hypothesis suggests transformation by entrapment of surface epithelium in inclusion cysts followed by stimulation of the entrapped epithelium by estrogen or estrogen precursors [27]. As with the incessant ovulation hypothesis, the recently introduced chronic inflammation model of carcinogenesis proposes that chronic exposure to external or endogenous triggers of immunity and persistent immune cells cause injury to surrounding epithelium, damage DNA through release of reactive oxygen species, or produce cytokines that promote proliferation [28].

The biological behavior of ovarian carcinoma is unique in that EOC metastasizes within the peritoneal cavity on organs within the peritoneal cavity including the omentum [29], penetrates the mesothelial layer and rarely deeper into the peritoneal layer [30]. Evidence is mounting that an inflammatory process contributes to tumor growth and metastasis to the peritoneum in EOC [31–35]. Epithelial ovarian cancer appears to be associated with inflammation, growth, differentiation, and signaling of ovarian tumor appear to be regulated by cytokines [36, 37]. Ovarian cancer risk factors that enhance local inflammation include endometriosis and pelvic inflammatory disease (PID) [27, 38]. The strong correlation between endometriosis and ovarian cancer also supports the chronic inflammation hypothesis [28]. A large population study from Taiwan found more than twofold increase in the risk for

development of ovarian cancer later in life and was correlated with the number of PID episodes [39]. Endometriosis, a condition associated with elevated inflammatory markers, has been found to increase risk of clear-cell, invasive endometrioid, and low-grade serous tumors [40, 41].

More than one third of ovarian cancer patients present with malignant ascites (peritoneal accumulation of fluid) at diagnosis; additionally, development of ascites is associated with chemo-resistant and recurrent disease [42, 43]. The concentration of inflammatory cytokines such as IL-1β, IL-6, IL-8 and IL-10 was shown to be significantly higher in the ascites of ovarian cancer patients compared to that present in the serum, and correlated with poor prognosis and response to therapy [44]. Among these cytokines, IL-6 and IL-10 have received the most attention due to their correlation with poor prognosis and response to therapy [45, 46].

Elevated IL-6 and C-reactive protein (CRP) levels are associated with a greater risk of ovarian cancer and support a role for inflammation, most likely subclinical, in initiating disease [28]. Systemic CRP levels in the blood rise rapidly in response to IL-6 released during local inflammatory processes. Higher CRP levels were found to be associated with ovarian cancer in samples collected an average of 6.4 years prior to diagnosis [47]. Elevated CRP levels was associated with higher overall EOC risk, and IL-6 and CRP may be associated with EOC risk among women with higher adipose tissue [48]. IL-6, as well as IL-2, IL-4, IL-12, and IL-13 levels were significantly associated with increased risk of developing epithelial ovarian cancer of combined histologies. The majority of cases were of the serous type and the results looked similar when they restricted analysis on the serous subtype [49]. Cumulatively, these results suggest that inflammation may precede ovarian cancer.

A cytokine network called the ‘TNF network’—consisting of TNFα, IL-6 and CXCL12, was recently identified in human HGSC and found to involve an autocrine network in which TNFα levels correlated with macrophage chemokine CXCL12 levels, TNFα levels correlated with IL-6 levels in human biopsies [50]. TNFα network pathway gene expression associated with genes involved in angiogenesis, inflammation, and leukocyte infiltrates. Ascites were obtained from HGSC patients who had been treated with the anti-TNFα antibody infliximab; interestingly, TNFα network gene sets were downregulated in these antibody-treated patients [50]. In summary, chronic inflammation in the reproductive tract is involved in ovarian cancer development. Models of ovarian cancer initiation likely are not exclusive and could act together to increase incidental ovarian cancer [28]. Unfortunately, to the best of our knowledge, no studies have been reported which directly compare in either a prospective cohort or case-control setting that smoldering subclinical inflammation drives development of ovarian cancer.

11.1.4 Obesity, Adipose Cells and Ovarian Cancer

11.1.4.1 Obesity and Inflammation

Obesity is implicated in ~20% of all cancer-related mortalities [51] and obese patients are more likely to have a poorer cancer prognosis, to develop metastases, and have a dampened response to anti-cancer therapies [52]. Obesity is intrinsically linked with metabolic syndrome, characterized by insulin resistance, hyperglycemia, hypertension, and dyslipidemia. Obese individuals are at a higher risk of developing a number of different cancers including ovarian, endometrial, breast (post-menopausal), gastric and colon cancers [53]. Recent links between inflammation and ovarian cancer may be associated with obesity and its consequences including metabolic syndrome [54]. Adipose tissue, and more specifically adipocytes, is playing a larger role in tumor initiation, growth and metastasis than previously thought. A role for adipose tissue in cancer is emerging based on two key observations: (1) epidemiologic studies have demonstrated an association between obesity and some cancers (e.g. esophageal and endometrial), and (2) adipocytes constitute a major component of the tumor microenvironment for breast and abdominally metastasizing cancers (ovarian, colon and gastric) promoting tumor growth [55, 56]. Many tumor types including ovarian cancer grow in the anatomical vicinity of adipose tissue.

White adipocytes are considered the dominant adipocyte subtype in adult humans. A critical step in white adipocyte physiology is the terminal differentiation of pre-adipocytes into adipocytes which allows increased storage of fatty acids, in the form of triacylglycerol (adipogenesis). Once terminally differentiated, the white adipocytes maintain energy homeostasis by storing and mobilizing lipids [55]. Excess triglyceride accumulation within adipocytes due to energy surplus results in adipocyte hypertrophy whereby adipocytes become dysfunctional. Hypertrophied adipocytes secrete increasing amounts of pro-inflammatory adipokine monocyte chemoattractant protein 1 (MCP-1), TNFα, IL-6, IL-8 and leptin [57] and results in the infiltration of lymphocytes, macrophages and stromal cells, significantly altering the adipose tissue microenvironment. In fact, macrophages and inflammatory cells may comprise up to 50% of the adipose tissue cellular content in obese subjects, compared to 5–10% in lean subjects [58]. Activated macrophages in adipose tissue are an essential contributor of pro-inflammatory cytokines and along with adipocytes contribute to chronic inflammation [59]. Therefore, a major feature of obesity is a state of chronic inflammation, heightened by increased circulating free fatty acids and recruitment of immune cells, in particular macrophages [60, 61]. Macrophages may be categorized as M1, an inflammatory phenotype, or M2, a scavenging/remodeling phenotype. Adipose tissue macrophages (ATMs) with an inflammatory M1-type phenotype have been identified in murine and human obesity [62].

11.1.4.2 Adipocytes and Ovarian Cancer

The biology of ovarian cancer is different from other cancers in that distant metastasis is rare and often confined to the peritoneal cavity [30]. The most common site of ovarian cancer metastasis is the omentum, a well vascularized adipose-rich tissue within the peritoneal cavity [63]. Human ovarian tumor cells quickly home to the omentum in an omental mouse model [29]. Primary human omental adipocytes induce ovarian cancer cell proliferation and invasion *in vitro* and ovarian cancer growth *in vivo* [64]. Adipocyte-secreted cytokines (IL-8 and IL-6) attract ovarian cancer cells to the omentum. In this manner, adipocytes engage in "metabolic coupling" with cancer cells and thereby promote tumor progression [65]. Mitochondrial metabolism in metastatic ovarian cancer cells is fostered, thereby protecting them from apoptotic cell death, as well as improving chemoresistance, and enhancing their colonization into macrometastatic lesions [64]. Leptin is an adipokine produced primarily by adipocytes and leptin-mediated signaling has been shown to promote ovarian cancer cell growth *in vitro* [66]. In a small ovarian cancer study, IL-10, leptin and osteoprotegerin (OPG) in the ascites were shown to be associated with shorter progression-free survival [67]. OPG inhibits TRAIL-induced apoptosis of ovarian cancer cells while IL-10 is known to inhibit T helper cell functions, hamper dendritic cell maturation, and inhibit T cell costimulatory molecules, suggesting that IL-10 in ascites may help tumor cells evade host immunological surveillance.

Given our understanding of the transition of a benign fibroblast to a cancer-associated fibroblast, it is reasonable to speculate that components of adipose tissue may be recruited by cancer cells and used to promote tumor growth. Several reports suggest that in the presence of cancer cells, adipocytes revert from mature, differentiated adipocytes into pre-adipocytes [55]. In the presence of cancer cells, adipocytes can also be reprogrammed into cancer-associated adipocytes (CAA). CAA secrete adipokines which stimulate the adhesion, migration, and invasion of tumor cells. Cancer cells and CAA also undergo a dynamic exchange of metabolites with CAA releasing fatty acids through lipolysis which are then transferred to cancer cells and used for energy production [55]. Adipose stromal cells (ASCs) also play an important regulatory role in cancer progression and metastasis by regulating systemic inflammation and tissue metabolism. ASCs (visceral and subcutaneous fat) facilitate migration of ovarian cancer cells via the IL-6/JAK2/STAT3 pathway further implicating IL-6 as a major player in ovarian cancer-related inflammatory pathways [68]. Targeting IL-6 with neutralizing antibody siltuximab inhibited cytokine production, angiogenesis and macrophage infiltration in preclinical studies and reduced IL-6-regulated levels of VEGF and macrophage chemokine CXCL12 levels in HGSC [69]. Similar to the TNFα network targeted clinical data with infliximab, targeting cytokines such as IL-6 is more likely to influence the tumor microenvironment than to kill malignant cells directly [50].

11.2 Microbiome and Inflammation

11.2.1 Microbiota Introduction

Microorganisms colonize tissues and organs such as the skin, gastrointestinal (GI), respiratory, and genitourinary systems. These microorganisms are generally called the human microbiota. The human microbiota consists of the commensal, symbiotic and pathogenic microorganisms found within and on the body and includes bacteria, archaea, protists, fungi, parasites and viruses [70, 71]. The skin and mucosal epithelia of humans and other mammals are permanently colonized by the microbiota and due to this life-long association, these microbes have an extensive influence over the physiology of their host organism. It is now becoming apparent that nearly all tissues and organ systems, whether in direct contact with the microbiota or in deeper host sites, are under microbiota influence. The microbial communities that reside within the human body contains at least 100 trillion (10^{14}) microbial cells composed of hundreds of microbial species [72–74], outnumbering eukaryotic cells 10:1 [75]. In addition to gut bacteria, virus and fungi live on and within different mucosal surfaces as well as within tissues [76, 77]. Indeed, the nasal cavity, oral cavity, esophagus, stomach, gut, vagina and skin are colonized by different microbes. High-throughput sequencing has revealed substantial intra-individual microbiome variation at different anatomical sites, and inter-individual variability for the same anatomical sites. However, higher level (e.g. phylum) taxonomic features display temporal (longitudinal) stability in individuals at specific anatomical sites. Such site-specific differences as well as observed conservation between human hosts provide an important framework to determine the biological and pathological significance of a particular microbiota composition [70].

The gut microbiota plays an important role in the maintenance of host health and keeping the colonic flora in a balanced state in which anti-inflammatory pathways are intact and pro-inflammatory pathways are kept in check. A preferred microbiota is that in which the so-called beneficial strains predominate over the potentially harmful species [78]. Healthy microbiota contains a balanced composition of diverse classes of bacteria. Commensals are permanent residents and are neutral to the host while symbionts are microbes with health-promoting functions. Pathobionts are also permanent residents with the potential to induce pathology; opportunistic organisms that cause rare and acute inflammation. During dysbiosis, there is an unnatural shift in the composition of the microbiota whereby either the numbers of symbionts are reduced and/or pathobionts are increased and may lead to non-specific inflammation which may predispose genetically susceptible people to inflammatory disease [79]. Symbionts include *Bifidobacteria, Lactobacilli, Faecalibacterium prausnitzii,* and *Bacteroides thetaiotaomicron*; pathobionts include certain *Bacteroides* species (e.g. *Bacteroides fragilis*) and *Clostridium difficile* (Firmicutes phyla) [80]. Symbiotic bacteria of the mammalian gut have been appreciated for the benefits they provide to the host: contribution to the development of the intestinal architecture, provision of essential nutrients, metabolism of

indigestible compounds, as well as defense against colonization by opportunistic pathogens (colonization resistance) [81].

Maturation of the immune system is dependent on exposure to the microbiota following birth [82]. In germ-free mice, which are protected from exposure to external microbes, spleens and peripheral lymph nodes are hypoplastic, mesenteric lymph nodes are mostly absent while primary immune organs, thymus and bone marrow, have normal appearance [83]. However, germ-free mice mount normal or heightened responses to nominal purified antigens but defective responses to pathogens due to deficient innate and APC functions [83–85]. Intestinal immune cells localize to Peyer's patches and mesenteric lymph nodes where T cells are antigen-stimulated and clonally expand (inductive sites) and migrate to effector sites such as the epithelium and underlying lamina propria [86]. Germ-free mice that lack microbiota have smaller Peyer's patches and reduced number of CD4+ T cells and IgA-producing plasma cells. The intestinal microbiota is therefore a key contributor to the proper structure of these sites [87].

Intestinal microbiota account for most of the human microbiota and is primarily composed of five bacterial phyla, Firmicutes, Bacteroidetes, Actinobacteria, Proteobacteria, and Fusobacteria. Firmicutes and Bacteroidetes predominate and represent ~90% of the total gut microbiota [88, 89]. However, species can vary greatly between individuals but are usually stable in a single individual over time [88]. Although there is high inter-individual variability in gut microbiota composition, a 'core gut microbiome' is shared by healthy adults and suggests a crucial role of the microbiota in the maintenance of optimal health [90]. The diet and environment are crucial to the acquisition of an adult-like microbiota and to the establishment of bacterial–host symbiosis. A high-fiber diet results in greater Bacteroidetes and a much lower abundance of Firmicutes (mainly composed of *Clostridium* species) in humans. Feeding germ-free mice (mice with no gut bacteria), colonized with human fecal matter from healthy people, with a high-fat "Western" diet compared to a low-fat plant-rich diet, significantly alters the microbiota composition, resulting in an increase in Firmicutes and decrease in Bacteroidetes phyla composition [89]. There is a correlation between dietary fiber content and diversity of gut microbial communities, as a low-fiber diet markedly reduces diversity of commensal microbes [91]. Although the composition of bacterial species may vary among individuals, a healthy gut microbiota presents diversity that is functionally redundant; more than one species may have the same overlapping metabolic functions [86]. This functional diversity confers resilience to our microbiota and helps with maintenance of homeostasis, maintaining a balance between pro- and anti-inflammatory mechanisms [92].

An aging population is now a common feature of western countries and an emerging phenomenon among developing countries [93, 94]. An immunological feature of the aging process is immunosenescence, characterized by persistent NF-kB-mediated inflammation and loss of naive CD4+ T cells [31]. Chronic activation of the innate and adaptive immune system is linked to immunosenescence [95]. Other than immunosenescence, aging is associated with a number of physiological and biological modifications including deterioration in dentition, salivary function,

digestion and intestinal transit time and may also affect the gut microbiota [96]. A controllable environmental factor is diet however, which has been shown to influence microbiota composition and health. The gut microbiota of the elderly (≥65 years) showed greater inter-individual variation than that of younger adults [97]. In 68% of the elderly individuals ($n = 161$), the microbiota was dominated by Bacteroidetes, with an average proportion of 57% across all elderly samples whereas Firmicutes had an average proportion of 40%. The proportions of some phyla and genera associated with disease or health also varied dramatically, including Proteobacteria, Actinobacteria, and *Faecalibacteria* [97].

In a follow-up study, fecal microbiota of the elderly (mean 78 years) was analyzed and it was found that microbial communities separated between the elderly depending on whether they lived in long-term residential care or were integrated into the community [98]. Long-term care elderly-derived microbiota had a higher proportion of phylum Bacteroidetes compared to a higher proportion of phylum Firmicutes in the community elderly. Young adult control microbiotas were more similar to the community elderly. Interestingly, clustering of cohorts by diet separated them by the same residence location and microbiota groupings. Four dietary groups (DGs) emerged: DG1 (low fat/high fiber) and DG2 (moderate fat/high fiber) that included 98% of the community elders, and DG3 (moderate fat/low fiber) and DG4 (high fat/low fiber) that included 83% of the long-term care elderly. Since in this study location largely determined diet, analysis by dietary groups rather than by residence location confirmed that both microbiota and diet were most diverse in DG1, and least diverse in DG3 and DG4 [98]. The separation of microbiota composition significantly correlated with measures of nutritional status and markers of inflammation among other variables. Markers of inflammation (serum TNFα, IL-6, IL-8, and CRP) were significantly higher in the elderly at long-term care rather than in community dwellers [98]. Lastly and importantly, the individual microbiota of people in long-term residential care was significantly less diverse than elderly that lived in the community [98]. Collectively, the data supports a role for the gut microbiota in varying rates of health decline upon aging and that diet can modulate the gut microbiota.

11.2.2 Gut Microbiota and Metabolism

Commensal bacteria are key regulators of digestion, a process that begins in the mouth and continues as ingested food and its digestive intermediates transit more than 20 ft (6 m) to the end of the adult human GI tract. Along the way, the digestive slurry is mixed with commensal bacteria, which is important for the extraction, synthesis and absorption of many nutrients and metabolites [99]. Core metabolic functions of microbiota include production of short chain fatty acid (SCFAs), amino acids, vitamins, bile acid biotransformation, hydrolysis and fermentation of non-digestible substrates [100]. In a westernized ‘high-fat’ diet, dietary polysaccharides

and proteins that escape digestion in the small intestine are fermented in the colon by the gut microbiota into SCFA consisting mainly of acetate (C2), propionate (C3) and butyrate (C4) [101, 102]. Butyrate and propionate can regulate intestinal physiology and immune function, while acetate acts as a substrate for lipogenesis and gluconeogenesis [103]. As described in the previous section, individual microbiota of the long-term care elderly was significantly less diverse than that of community dwellers and loss of community-associated microbiota correlated with increased frailty [98]. In terms of metabolism, butyrate, acetate and propionate were found in higher levels in community elderly compared to long-stay elderly. Interestingly, metagenomes were searched for key microbial genes in butyrate, acetate and propionate production, revealing significantly higher gene counts for butyrate- and acetate-producing enzymes in community elderly compared to long-stay care elderly [98]. Recently, key roles for these metabolites have been identified in regulating immune function in the periphery, oral tolerance and resolution of inflammation, and also for regulating the inflammatory output of adipose tissue [104]. As carbohydrates become depleted, digested foodstuff moves distally through the colon, the gut microbiota switches to other substrates, notably protein or amino acids. Fermentation of amino acids, besides liberating beneficial SCFAs, produces a range of potentially harmful compounds, some of which have been implicated in initiation or progression of gut permeability, DNA damage, and IBD [105]. SCFAs are absorbed and used as nutrient sources by epithelial cells and distributed throughout the body, but the effect of SCFAs extend beyond nutrition and can have effects on immune cells as discussed in the next section. These metabolites have a well characterized anti-inflammatory effect, on both gut epithelial and immune cells, as reviewed elsewhere [106, 107].

Bile acids are a family of cholesterol-derived molecules that solubilize dietary fat in the small intestine to support the digestion and absorption of fat. In addition to their roles in regulating digestion, bile acids act as signaling molecules that regulate metabolic homeostasis [108, 109]. Commensal bacteria are required for the production of bile acids which have anti-inflammatory properties. Some bile acids can regulate the function of immune cells via the G protein-coupled bile acid receptor 1 (GPBAR1; also known as TGR5 and membrane-type receptor for bile acids, M-BAR) and the nuclear receptor subfamily 1, group H, member 4 (NR1H4; also known as farnesoid X receptor, FXR), both of which are highly expressed in monocytes and macrophages as well as other immune cell types [108, 109].

Recent studies on the modulation of immunity against infection by microbiota have provided insight into how commensals regulate systemic immunity. Germ-free or antibiotic-treated mice have defective myelopoiesis and impaired neutrophil homeostasis with an increased susceptibility to late-onset sepsis [110]. Defective myelopoiesis also results in germ-free mice unable to resist acute infection with *Listeria monocytogenes*, however, mice have an enhanced adaptive immune response to vaccination with an attenuated *L. monocytogenes* strain [111, 112].

11.2.3 Effects of Gut Microbiota on Immune Cells

The microbiota in humans begins to develop after birth, diversifies as the infant grows and by adulthood, a stable community has evolved, dominated by bacterial phyla Bacteroidetes and Firmicutes, although it varies widely between healthy individuals [113]. The microbiota is modulated by factors including gestational age, mode of delivery (natural or by Caesarean section), diet (breastfeeding or infant formula), hygiene, and antibiotic exposure [114]. Today, it is well established that gut commensal bacteria profoundly shapes mammalian immunity [87, 115], and the immune system in turn shapes the composition of the microbiota [116]. Early studies have identified impaired host immune responses to pathogens in mice treated with antibiotics or raised under germ-free conditions [117–119]. Mice given drinking water with a cocktail of oral antibiotics (ampicillin, gentamicin, metronidazole, neomycin, vancomycin) had impaired innate and adaptive antiviral immune responses and substantially delayed viral clearance after exposure to systemic LCMV or mucosal influenza virus [120]. Macrophages isolated from treated mice displayed decreased expression of genes associated with antiviral immunity and exhibited defective responses to type I/II interferons (IFN) and concomitant impaired ability to limit viral replication [120]. Therefore, tonic signaling (calibration of the activation threshold) was dependent on commensal-derived signals to maintain the fitness of antiviral pathways in macrophages.

The immunological impact of microbiota composition is gaining increased recognition as a pivotal player in immune system development and T cell differentiation [121, 122]. Th17 cells secrete IL-17A and IL-17F and have significant roles in protecting the host from bacterial and fungal infections, particularly at mucosal surfaces. Th17 cells also have potent inflammatory potential, and are key mediators of autoimmune disease [123, 124]. Notably and surprisingly, at steady state, most IFNγ (Th1) T cells and IL-17 (Th17) are found in the GI tract and develop from signals derived from the microbiota, as detailed below [87, 125, 126].

The microbiota stimulates innate responses translates into its important role in the induction of adaptive immunity. Mice from germ-free mice have lower numbers and malfunctioning IL-17-producing CD4+ T cells as well regulatory T cells (Treg) [127, 128]. Different bacterial species induce distinct immune cell populations that can play pro- and anti-inflammatory roles, and thus the composition of the microbiota determines, in part, the level of resistance to infection and susceptibility to inflammatory diseases [129]. Chronic colonization with enterotoxigenic *Bacteroides fragilis* induces STAT3 signaling characterized by a Th17 response that leads to colonic hyperplasia and increased tumorigenesis in an intestinal neoplasia mouse model [130]. Th17 cells produce other cytokines besides IL-17, such as IL-22, another cytokine linked to human colon cancer by activation of STAT3 [131]. In contrast, *B. fragilis* induces immune tolerance by activating Treg and the production of IL-10 [79, 132]. Treg cells expressing transcription factor Foxp3 have a key role in limiting inflammatory responses in the intestine. Specific bacteria such as *Clostridia* help drive intestinal Treg expansion and development [121], which can

suppress inflammatory disease in mouse models. Induced Treg suppress excessive immune responses [133].

A full understanding of how the commensal microbiota impacts the host immune system remains incomplete. Initially it was known that CD4+ T cells acquire distinct functional properties in response to signals from commensal and pathogenic microbe-activated cells of the innate immune system [134]. The relevance of the gut microbiota in immune system development as well T cell differentiation is exemplified by the segmented filamentous bacterium (SFB), a gram-positive *Clostridia*-related species, and largely recapitulates the coordinated maturation of T cell responses induced by the entire mouse microbiota [125]. Notably, at steady state, most IL-17 (Th17) is found in the GI tract and develops from signals derived from SFB [126]. Colonization of the small intestine of mice with SFB is sufficient to induce the appearance of Th17 cells, i.e., CD4+ T helper cells that produce IL-17 and IL-22 [126].

As briefly introduced previously, SCFAs (mainly acetate, propionate and butyrate) generated by the gut microbiota has anti-inflammatory potential by modulating cells of the immune system. Butyrate and propionate can regulate intestinal physiology and immune function [103]. In addition to acting as a local nutrient source for colonocytes, butyrate has also been shown *in vitro* and *in vivo* to regulate energy homeostasis by stimulating leptin production in adipocytes, as well as inducing glucagon-like peptide-1 (GLP-1) secretion by intestinal enteroendocrine L cells [135]. Key roles for these metabolites have been identified in regulating immune function in the periphery, directing appropriate immune responses, oral tolerance and resolution of inflammation [104]. Specifically, butyrate and propionate (supplied in drinking water) facilitated extrathymic generation of Treg cells in mice, suggesting that bacterial metabolites mediate communication between the commensal microbiota and the immune system, affecting the balance between pro- and anti-inflammatory mechanisms (Fig. 11.1) [104].

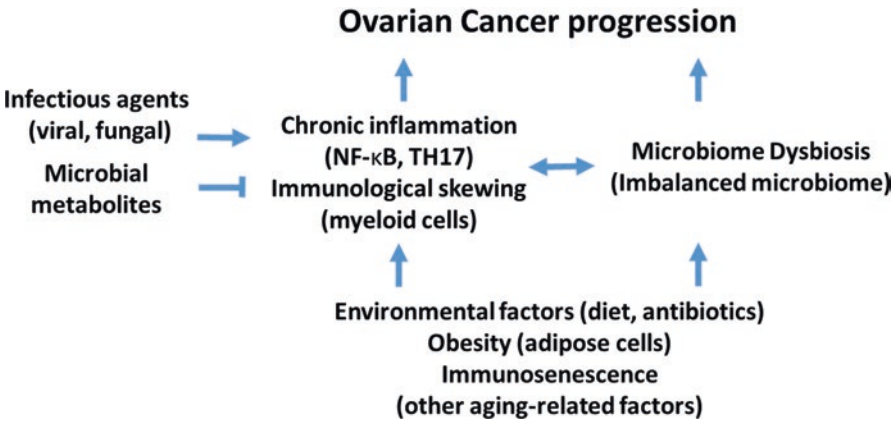

Fig. 11.1 Links between microbiome dysbiosis, chronic inflammation and ovarian cancer

Butyrate regulates neutrophil function and migration, inhibits inflammatory cytokine-induced expression of vascular cell adhesion molecule-1, increases expression of tight junction proteins in colon epithelia, and exhibits anti-inflammatory effects by reducing cytokine and chemokine release from human immune cells [116]. SCFA can also directly impact functionality of macrophage and DCs; propionate was shown to affect mouse DCs and macrophage biology in the bone marrow and impaired ability of DCs to promote Th2 cell effector function in the lungs [91]. Butyrate and propionate treatment of human DCs significantly reduced lipopolysaccharide (LPS)-induced IL-6 mRNA and IL-12 gene expression and modulates leukocyte trafficking, as SCFA strongly reduced the release of several pro-inflammatory chemokines [136]. These findings support the concept that bacterial metabolites far from the site of their production can differentially modulate APC activity and effector function.

Bile acids appear to regulate the function of at least some immune cell types through GPBAR1 and NR1H4, both of which lead to the inhibition of NF-κB-dependent expression of pro-inflammatory genes [99]. In macrophages and monocytes, bile acid signaling via these receptors is linked to a common anti-inflammatory response involving the inhibition of NF-κB activity and repression of NF-κB-dependent transcription [137, 138]. The role of commensal bacteria in the production of bile acids and the anti-inflammatory effects of bile acids in some cell types has been implicated in diseases such as IBD and atherosclerosis [137, 138]. The bile acid-mediated decrease in NF-κB activity in macrophages and monocytes is associated with the impaired antiviral immunity observed in germ-free mice or mice with experimentally-altered composition of commensal bacteria [120, 139]. Macrophages from germ-free and antibiotic-treated mice have lower NF-κB-dependent gene expression and IFN responses in association with diminished CD8+ T cell and NKT cell function as well as increased susceptibility to viral infection [120, 139]. Cumulatively, these studies suggest that commensal microbiota may provide instructive tonic signals via SCFA and bile acids that support the proper functioning of innate immune cells and the coordination of adaptive immune responses [120, 139].

Gut microbiota regulate natural killer (NK) and APC function. NK cells, residing in non-mucosal lymphoid organs of germ-free mice, could not be primed to mount effective antiviral immunity. Adoptive transfer experiments revealed that this is not an NK cell-intrinsic defect but rather reflects impaired priming of NK cells by APC [139]. APC are mononuclear phagocytic cells such as macrophages and DCs that express PRR, the ligation of which leads to the induction of an inflammatory gene expression program required for an effective response against pathogens. In non-mucosal lymphoid organs (spleen and peripheral lymph nodes), the total numbers of macrophages and both migratory and resident DC subpopulations are not affected in germ-free mice. However, macrophages and DCs from germ-free mice failed to produce IFN-I in response to microbial ligands or viral infection [139]. DC from germ-free mice fail to respond to the TLR3-ligand poly(I:C) and to LPS with production of cytokines such as type I IFN, IL-12, IL-6 and TNFα [140]. In microbiome-constricted mice, in spleen and mesenteric lymph nodes, there was an increased

prevalence of mature myeloid DC, producing greater amounts of IL-12, and concomitantly greater numbers of IFNγ+ CD8+ T cells. Plasmacytoid DC were selectively deficient in these mice and was reversed by depletion of CD8+ T cells. Therefore, the microbiota shapes the systemic DC population in a process involving recruitment of cytolytic CD8+ T cells [140]. Lastly, crosstalk between bacteria in the form of quorum sensing peptides may participate in tuning DC programs regulating T cell effector function; for example, by driving DC IL-12 production [141].

Recent studies on the modulation of immunity against infection by microbiota have provided insight into how commensals regulate systemic immunity. Germ-free or antibiotic-treated mice have defective myelopoiesis and impaired neutrophil homeostasis with an increased susceptibility to late-onset sepsis [110]. Defective myelopoiesis also results in germ-free mice unable to resist acute infection with *Listeria monocytogenes*, however, mice have an enhanced adaptive immune response to vaccination with an attenuated *L. monocytogenes* strain [111, 112]. In summary, the microbiota regulates immune homeostasis both at the local mucosal level and systemically acting primarily although not exclusively at the cellular level of myeloid-derived APC cells.

11.2.4 Gut Microbiota and Obesity

The first proof of concept regarding the role of gut microbiota in the modulation of body fat was demonstrated with germ-free mice; when fed a standard chow diet, these mice gain less body fat than conventional mice despite increased food intake [142]. In a process called conventionalization, germ-free mice were given a suspension of cecal contents onto their fur from normal donor mice that harbored a microbiota since birth; these germ-free mice subsequently saw a dramatic increase (57%) in their total body fat content [142]. This hallmark study demonstrated a relationship between the gut microbiota and development of an obese phenotype. These findings suggested that microbiota of obese individuals may be more efficient at extracting energy from a given diet than the microbiota of lean individuals. Utilizing the obese mouse model with a mutation in the leptin gene, it was shown that the obese microbiome has an increased capacity to harvest energy from the diet [143]. Furthermore, this trait is transmissible: colonization of germ-free mice with an 'obese microbiota' (by gavage) results in a significantly greater increase in total body fat than colonization with a 'lean microbiota.' These results identify the gut microbiota as a contributing factor to the pathophysiology of obesity [143]. An increase in the Firmicutes/Bacteroidetes ratio is associated with the microbiota of obese mice [143, 144]; similar data in a human dietary intervention study demonstrated that weight loss of obese individuals (body mass index, BMI > 30) was accompanied by an increase in the relative abundance of Bacteroidetes [145]. Lastly, the cecum from obese mice has an increased concentration of the major fermentation end-products butyrate and acetate, consistent with the fact that many Firmicutes are butyrate producers [143].

An increase in the Firmicutes/Bacteroidetes ratio is associated with systemic and adipose tissue inflammation and development of metabolic syndrome, (obesity, insulin resistance and type 2 diabetes) [92], although this observation is controversial due to heterogeneity among human subjects with respect to genotype and lifestyle [145–147]. Obesity is also associated with an overall reduction in gut bacterial diversity [143] and decreased bacterial richness has been linked to elevated systemic inflammation, measured by CRP and white blood cell counts [148]. High-fat feeding is accompanied by impairments in gut barrier function and higher plasma levels of LPS, a component of the outer membrane of Gram-negative bacteria [149]. LPS, acting as a trigger, has previously been shown to induce metabolic endotoxemia, characterized in part by pro-inflammatory cytokine expression and elevated adipose tissue macrophages (ATMs) infiltration [150]. Increased *systemic* inflammation is observed in high-fat diet fed mice, and diet-induced inflammation can be completely prevented by treatment with a broad-spectrum antibiotic [149].

Dysbiosis present in obese individuals alters the gut epithelial barrier, making it more permeable to microbial products that activate immune cells in the lamina propria, reaching the liver via the portal circulation, and contributes to the production of pro-inflammatory cytokines, such as TNFα and IL-6 [151]. The gut of individuals with OAMD (obesity-associated metabolic disorder) is believed to harbor an inflammation-associated microbiome, with a lower potential for butyrate production and reduced bacterial diversity and/or gene richness [102]. Although the main cause of OAMD is excess caloric intake compared with expenditure, differences in gut microbial ecology might be an important mediator and a new therapeutic target or a biomarker to predict metabolic dysfunction/obesity in later life [102]. Several mechanisms have been suggested to explain the role of gut microbiota in the etiology of obesity such as SCFA production, bile acid metabolism and chronic low-grade inflammation [101]. Cumulatively, gut dysbiosis and impaired barrier function associated with obesity can induce adipose tissue inflammation leading to chronic systemic inflammation. Given the known role this type of inflammation plays in the progression of many cancers, there is some probability that obesity-induced perturbations of the gut microbiota are a contributing factor in the obesity-cancer link (Fig. 11.1).

11.2.5 Gut Microbiota Effects in Preclinical Models and Human Immunotherapy

An important consideration to understand the role of the microbiota in health and disease is highlighted by environmental differences in preclinical mouse habitation from different vendors. Differences in the composition of commensal microbiota may influence experimental variation across different laboratories and even within the same laboratory. Important factors that impact microbiota-dependent mechanisms in multiple ways include the hygiene of the housing facility, nature of the diet

and the pH of drinking water [152, 153]. Genetically similar C57BL/6 mice derived from two different mouse facilities, Jackson Laboratory (JAX) and Taconic Farms (TAC), have been shown to differ in their commensal microbes [126] and impacts tumor growth. This observation has provided a model on how intersubject heterogeneity in cancer development might be impacted by the microbiota [154]. TLR5-deficient animals bearing tumor is an example in which genetically identical mice have yielded differences in intestinal inflammation and metabolic syndrome in different facilities. PCR amplification of 16S ribosomal RNA at tumor, lymphatic or other non-mucosal locations was comparable to germ-free wild type tumor-free mice [155]. Surprisingly, the same TLR5-deficient mice housed at different facilities exhibited metabolic syndrome, the presence of colitis and increased levels of IL-1β within the colons of these mice [156, 157].

For decades, cancer therapy was based on surgical resection to decrease tumor burden, followed by chemotherapy and/or radiation to target rapidly growing tumor cells, while mostly sparing quiescent normal tissues [158]. The field of adoptive cell therapy (ACT) for solid tumors was established with the discovery that tumor-infiltrating lymphocytes could be expanded and used to treat patients with metastatic melanoma, with objective response rates of 50% with some patients with durable remissions [159, 160]. ACT with addition of total body irradiation (TBI) with a preparative regimen of cyclophosphamide and fludarabine is associated with decreased Treg reconstitution, suggesting a possible benefit with increased intensity lymphoconditioning [161].

The gut microbiota plays a beneficial role in ACT therapy with the use of TBI which promotes an LPS-TLR4-dependent activation of APC facilitating the efficacy of ACT [162]. Lymphodepletion with TBI increases the efficacy of ACT tumor-specific CD8+ T cells by depleting inhibitory lymphocytes and increasing cytokine levels. TBI also augments the function of ACT CD8+ T cells in immunodeficient mice, suggesting another TBI mechanism of action. Commensal gut microflora in the mesenteric lymph nodes and elevated LPS levels in the sera of irradiated mice correlated with increased DC activation and increased levels of systemic inflammatory cytokines. Disruption of the homeostatic balance between the host and the microbiota via gut permeability and microbial LPS translocation can enhance cell-based tumor immunotherapy [162].

A similar mechanism is proposed to explain the protective role of the microbiota in the context of chemotherapy. Cyclophosphamide, a clinically important cancer drug, leads to intestinal damage, bacterial dysbiosis and translocation and induction of anti-commensal Th17 responses that collectively contributes to the antitumor response [163]. Cyclophosphamide administration was found to increase gut epithelial permeability, alter the intestinal microbiota composition, and increase bacterial translocation from the intestinal lumen to secondary lymphoid organs, which resulted in enhanced populations of CD4+ T cells that expressed both IFNγ and IL-17. Responses of a mastocytoma and sarcoma to cyclophosphamide chemotherapy were reduced in mice with an antibiotic-damaged microbiota, a defect that was corrected by adoptive transfer of IFNγ/IL-17-producing T cells [163].

Treatment with antibiotic vancomycin improved immunotherapy with ACT against tumor growth. Gram-positive bacteria depletion with vancomycin induced an increase in systemic CD8+ DC, these DC sustained systemic expansion of adoptively transfer antitumor T cells [164]. Adoptive T cell transfer of antigen-specific T cells were injected into mice from two vendors, Harlan (HAR) and Jackson Labs (JAX). ACT had a significant impact on pre-established tumor progression in both sets of mice; tumor growth in HAR mice was almost completely abrogated, while in JAX mice ACT was significantly less effective. Vancomycin abrogated the difference of ACT efficacy between mice obtained from different vendors. The difference in ACT efficacy was attributable primarily to the presence of many Bacteroidetes taxa in HAR mice, while the JAX mice were dominated by a single Bacteroidetes taxon. Vancomycin induced tumor microenvironment remodeling more supportive for T cell infiltration and cytolytic activity, and increased the number of CD8+ DC in spleen and draining lymph nodes. Antibiotic treatment did not improve ACT efficacy when IL-12 deficient mice were tested as there were no differences in tumor progression or T cell infiltration, supporting a role for IL-12 in this study[164].

Recent studies in murine models have also implicated the gut microbiota in responses to cancer chemotherapy by another distinct immunologically-mediated mechanism [165]. Response to immunotherapy for several cancers (lymphoma, colon carcinoma, and melanoma) were reduced in mice with absent or antibiotic-depleted microbiota, as reflected by reduced TNFα production and reactive oxygen species by tumor-infiltrating myeloid cells. Response of lymphoma to platinum chemotherapy was reduced in the absence of a complete microbiota [165]. Remarkably, tumor control was associated with the presence of defined commensal species such as *Alistipes shahii* [165]. Therefore, specific bacterial species of the microbiota, can control various aspects of immunity associated with antitumor responses, an effect that has profound clinical implications. These results suggest that the inflammatory response that follows cancer therapy, which is strongly enhanced by the translocating microbiota, contributes to tumor eradication through the upregulation of IL-17 and TNFα [163, 165]. There is data however to suggest the opposite in both mouse models and humans for IL-17 and TNFα, suggesting that cytokines modulated by the gut microbiota can have opposing effects on tumor growth and the outcome of cancer therapy, all of which need to be carefully considered when translating data from mouse models to patients with cancer [166]. It is unlikely that patients with cancer will have a grossly depleted gut microbiota, so it is debatable whether these studies can be applied in the clinic in the near future, but detailed studies of specific antibiotics and their effects on the microbiota are ongoing [166].

Modulating microbial activities may boost drug efficacy or alleviate toxicity, two key aspects of chemotherapeutic treatment. Targeting microbial activities has been shown to attenuate irinotecan-associated gastrointestinal toxicity in mice [167]. Irinotecan, a commonly used chemotherapy for colorectal cancer, can cause both immune suppression and diarrhea. However, in some patients, irinotecan can cause a severe and refractory diarrhea that requires hospitalization and limits the drug's subsequent dosing and usage. Irinotecan is a prodrug and is converted to the active

SN-38. Within the intestinal lumen, bacterial b-glucuronidase can liberate SN-38. Thus, the levels of intestinal bacterial b-glucuronidase and subsequent degree of intestinal epithelial SN-38 exposure influence the drug toxicity for patients. The identification of compounds that can improve drug efficacy and reduce toxicity represents an exciting direction for microbiota-based oncology therapeutics [168].

B16 melanoma implanted subcutaneously was found to grow more aggressively in mice obtained from TAC compared to JAX facilities and it was found to be immune-mediated in that antitumor T cell responses and CD8+ T cell tumor infiltration in JAX mice was greater than TAC mice [154]. These differences were eliminated upon cohousing or after fecal transfer, with the dominant JAX mice phenotype prevailing, suggesting that these mice had gut microbiota that impacted antitumor immunity. JAX fecal material alone or in combination with checkpoint inhibitor PD-L1 antibody was administered to TAC mice bearing established tumors. Transfer of JAX fecal material alone resulted in significantly slower tumor growth, with increased tumor-specific T cell responses and enhanced infiltration of these T cells into the tumor. The combination with PD-L1 antibody further inhibited tumor growth. PD-L1 antibody therapy alone was significantly more efficacious in JAX mice compared with TAC mice. These results suggest that the gut microbiota can impact immunotherapy and influence spontaneous antitumor responses [154]. Fecal bacteria was analyzed over time using 16S ribosomal RNA and it was found that *Bifidobacterium* showed a positive association with antitumor T cell responses. *Bifidobacterium* was fed orally to TAC mice and displayed significantly improved tumor control in comparison with non-fed TAC mice and by robust induction of systemic tumor-specific T cells and increase in antigen-specific CD8+ T cells within the tumor. This therapeutic effect of *Bifidobacterium* feeding was abrogated in CD8-depleted mice, which indicated that the mechanism was not direct but rather through host antitumor T cell responses. Lastly, a greater percentage of MHC-II high DCs was found in the tumors of JAX and *Bifidobacterium*-treated TAC mice [154]. Modulating microbial activities may boost drug efficacy or alleviate toxicity, two key aspects of chemotherapeutic treatment.

A landmark study showed that the presence of intratumoral CD3+ lymphocytes correlates with improved clinical outcome in advanced ovarian carcinoma. The 5-year overall survival rate was 38% among untreated patients whose tumors contained T cells and almost 5% among patients whose tumors contained no T cells [169]. Five-year overall survival further improved with the presence of intratumoral CD3+ T cells after surgical debulking and adjuvant chemotherapy and suggests that ACT is a viable immunotherapy in ovarian cancer treatment [169]. Immune checkpoint CTL-associated antigen 4 (CTLA-4) and programmed death-ligand 1 (PL-L1) have recently generated great clinical interest. PD-L1 is expressed on activated T and B cells, macrophages and DCs as well as cancer cells [170]. The engagement of PD-L1 with the PD1 receptor on T cells results in decreased effector T cell function and increased apoptosis of T cells [171, 172].

Inhibition of the PD1 pathway has been shown to be effective in restoring T cell function and immune responses against cancers [173]. Checkpoint inhibitors ipilimumab (IPI), nivolumab and pembrolizumab have yielded exciting clinical

results to date with durable responses in selected patients in various cancers [174–176]. In preclinical ovarian cancer models, checkpoint inhibitor therapy was investigated with the rationale that TILs present in tumors are in a functionally suppressive microenvironment that can be ameliorated with inhibition of immune checkpoints. CD8+ T cells restrict tumor progression, while Treg, by inhibiting CD8+ T cells, facilitate tumor progression, relying on PD-1, PD-L1 or CTLA-4 to carry out these functions. In preclinical studies, double-positive (PD-1 + CTLA-4+) CD8+ TIL have characteristics of more severe dysfunction than single-positive (PD-1+ or CTLA-4+) TIL, including an inability to proliferate and secrete effector cytokines. Blockade of both PD-1 and CTLA-4 resulted in reversal of CD8+ TIL dysfunction and led to tumor rejection in the murine ID8-VEGF ovarian carcinoma model [177]. Double blockade was associated with increased proliferation of antigen-specific effector CD8+ and CD4+ T cells, antigen-specific cytokine release, inhibition of suppressive functions of Treg, and upregulation of key signaling molecules critical for T cell function [177].

In ovarian tumors, in addition to immunosuppressive Treg, cells of the myeloid lineage are major determinants of immune suppression. These include TAMs, MDSC and immature/tolerogenic DCs. Using the ID8 syngeneic mouse model of epithelial ovarian cancer, it was shown that T-cell dysfunction can be reversed by targeting the PD-1 pathway simultaneously in all these cell types [178]. Expansion of ovarian antigen-specific CD8+ TILs was dependent on the amount of PD-L1 signaling by tumor cells, tumor-derived myeloid cells, and Treg. Cumulatively, these studies show that the PD-1/PD-L1 pathway is a key pathway in maintaining an immunosuppressive tumor microenvironment and inhibition of this pathway inhibits suppressive lymphocytes as well as myeloid suppressive cells and augments effector T cell activity. Evidence suggests that the gut microbiota also impacts human checkpoint inhibitor immunotherapy. Gut microbes have ascended to prominence as key modulators of host immunity in mouse models, suggesting possible influence on the outcome of cancer immunotherapy [179]. The antitumor effects of CTLA-4 blockade was found to be impacted by distinct *Bacteroides* species. In mice and patients, T cell responses specific for *B. thetaiotaomicron* or *B. fragilis* were associated with the efficacy of CTLA-4 blockade [180]. Tumor-bearing mice that were antibiotic-treated or germ-free mice did not respond to CTLA-4 blockade. Oral gavage with *B. fragilis*, immunization with *B. fragilis* polysaccharides, or by adoptive transfer of *B. fragilis*–specific T cells, all restored CTLA-4 blockade efficacy. Fecal microbial transplantation (FMT) from humans to mice confirmed that treatment of melanoma patients with CTLA-4 blockade favored the outgrowth of *B. fragilis* with anticancer properties. This microbiota-dependent mechanism depended on the mobilization of lamina propria CD11b+ DC that can process polysaccharides and then mount IL-12-dependent TH1 immune responses against *B. fragilis* capsular polysaccharides [180].

Human CTLA-4 blockade with IPI is associated with immune-mediated colitis and is observed in mice as well as patients [174, 180]. A prospective study of patients with melanoma undergoing IPI treatment was performed to understand the mechanism involved in iatrogenic (therapy-induced) colitis and an association was

found between the pre-inflammation fecal microbiota and microbiota composition with subsequent colitis development [181]. Specifically, increased bacteria from the Bacteroidetes phylum is correlated with resistance to antibody therapy-induced colitis [181], consistent with a proposed immunomodulatory role of these commensal bacteria. Bacteroidetes represents one of the major phyla of the human gut microbiota and its members can limit inflammation by stimulating Treg differentiation [182, 183]. IPI indirectly alters the gut bacteria to favor enrichment of *Bacteroides* species, possibly by promoting deterioration of the epithelial barrier via activation of local lymphocytes. These bacteria then promote the activation of DCs, which present tumor antigens to prime and support antitumor T cell responses [179]. Thus, the gut microbiota can affect cancer therapy outcomes, albeit therapy-induced adverse events, and suggests the possibility to use Bacteroidetes to prevent therapy-induced colitis. Understanding the mechanisms involved by gut microbiota in regulating the efficacy of therapy likely can be exploited to maximize these immunotherapies in the future [184].

Interindividual differences in the microbiota likely accounts for the significant heterogeneity in therapeutic and immunopathologic responses to immune checkpoint therapies [185]. Variability in individuals over time is consistently lower than interindividual variation, both in organismal composition and in metabolic function [88]. New insights could potentially improve the therapeutic coverage of checkpoint inhibitors, and potentially limit their immune-mediated toxicity, through the use of adjunctive "oncomicrobiotics" that indirectly promote beneficial immune responses through optimizing the gut microbiota [185]. Mechanisms underlying IBD and anti-CTLA-4-induced colitis stresses the crucial role of gut microbiota and of Treg in the genesis of both iatrogenic and spontaneous IBD as recently reviewed elsewhere [174].

Antibiotics compromises the efficacy of certain anti-cancer treatments, implicating commensal microbes as partners driving systemic inflammation, with the caveat that each vaccine may have a specific mechanism: oxaliplatin and CpG treatment effectiveness requires gut bacteria that generate myeloid-derived TNFα, while cyclophosphamide treatment requires commensal-derived IL-17 and Th1 responses [163, 165]. Cyclophosphamide (CTX) is considered an immunomodulatory anticancer compound. Antitumoral efficacy of CTX relies on two gut commensal species, *Enterococcus hirae* and *Barnesiella intestinihominis.* These two bacteria changed the tumor microenvironment, reducing Treg and stimulating cognate antitumor CTL responses [186]. *E. hirae* translocated from the small intestine to secondary lymphoid organs, induces systemic Th17 cell responses associated with tumor antigen-specific, MHC I-restricted CTL and increased the intratumoral CTL/Treg cell ratio. CD4+ T cell responses against *E. hirae* are associated with survival in ovarian cancer patients [186]. *B. intestinihominis* boosts systemic polyfunctional Tc1 and Th1 cell responses and reinstate intratumoral IFNγ-producing γδ T cells. Both commensals reduced Treg cells in the tumor microenvironment (Foxp3 T regs and/or γδ T cells). *E. hirae* and *B. intestinihominis* specific-memory Th1 cell immune responses selectively predicted longer progression free survival in ovarian cancer patients (n = 13) treated with chemo-immunotherapy (metronomic CTX)

[186]. Lastly, intestinal epithelial cells (IEC) NOD2 immune sensors represent "gut immune checkpoints" restricting the immunogenicity of distinct Gram-positive and Gram-negative bacteria. These two immunogenic commensals are kept in check by intestinal NOD2 receptors, limiting their direct pro-apoptotic effects on epithelial cells and their accumulation *in vivo* [186]. Microbe specific-memory CD4 Th1 cell immune responses selectively predicted longer progression free survival in ovarian cancer patients treated with metronomic CTX also warrants further inquiry.

11.3 The Microbiome and Ovarian Cancer

11.3.1 Microbiome Signatures Associated with Cancer

Genetic and environmental factors disrupting the healthy relationship between hosts and microbiomes can generate dysbiosis and promote cancer development. Lifestyle, diet, and early exposure to antibiotics have been recognized as major players in determining the microbiome composition. Potential factors that can promote or inhibit microbial dysbiosis include diet- and microbial-derived metabolites, generating inflammatory mediators and a pro-inflammatory state or inhibiting inflammation (Fig. 11.1). Although inflammatory, infectious and neoplastic diseases are often considered categorically distinct processes, evidence has shown significant overlap between them. Infectious agents are one of the main contributors to cancer development. In fact, it is estimated that 15% of worldwide cancer is of infectious nature, with human papillomavirus, hepatitis B virus, hepatitis C virus, human herpesvirus-8, and *Helicobacter pylori* recognized as the definitive cause of cervical cancer, liver cancer, Kaposi's sarcoma and stomach cancer/lymphoma, respectively [187]. The linkage of infection with some biological agents and carcinogenesis in humans started more than a century ago with Francis Peyton Rous [188]. Eleven biological agents have been identified as group 1 carcinogens by the International Agency for Research on Cancer (IARC) [189] and has been reviewed elsewhere [190]. A better understanding of the role of infectious agents in the etiology of cancer is an essential element for precision medicine because such cancers are theoretically preventable by proper vaccination or early treatment of infection [191].

Infectious agents can be direct carcinogens, such as HTLV- 1 and the KSHV, which express viral oncogenes that directly contribute to cancer cell transformation, or indirect carcinogens by causing chronic inflammation, which eventually leads to carcinogenic mutations in host cells, such as *H. pylori*, the major cause of gastric carcinogenesis. In addition, carcinogenesis can result from the interaction of multiple risk factors including those related to the infectious agent itself (virulence factors or variants), host-related factors (gene polymorphisms and immune system status) and environmental aspects (smoking, chemicals, ionizing radiation, immunosuppressive drugs, or another infection that may lead to reactivation of latent oncogenic viruses such as EBV or KSHV) [191]. Given that the human microbiota

contains endogenous viral component as well as microbial phyla in the healthy state, it is likely that their association with cancer is underestimated due to heretofore unrecognized infection [192]. For example, persistent infection by one or more infectious agents, resulting in inflammation or alteration of cellular processes, may be involved in the carcinogenic process [193]. Alternatively, the tumor microenvironment may provide a specialized niche in which these organisms can persist in a way that is difficult in normal tissue. In either case, the identification of unique microbial signatures associated with specific cancers is essential for our understanding of the interplay between the microbiome and cancer, knowledge that can lead to diagnostic and prognostic utility.

Human tumor viruses belong to two virus families, the RNA virus families (e.g., Retroviridae, Flaviviridae) and the DNA virus families (e.g., Herpesviridae, Papillomaviridae, Hepadnaviridae). Viruses associated with different types of human malignancies include HPV (cervical cancer, skin cancer, head and neck cancers), HHV-8 (Kaposi's sarcoma) and HBV and HCV (hepatocellular carcinoma) [194]. There are other viruses which can potentially contribute to human cancers including simian vacuolating virus 40 (brain cancer, cancer, and mesothelioma), BK virus (prostate cancer), JC virus (brain cancer), Torque teno virus (gastrointestinal cancer, lung cancer, breast cancer, and myeloma) human endogenous retroviruses (breast cancer, ovarian cancer, and melanoma) and human mammary tumor virus (breast cancer) [194].

Triple negative breast cancer (TNBC) lacks targetable receptors such as the endocrine receptors for progesterone, and estrogen as well as the EGFR receptor HER2, and is the most aggressive form of the disease [195]. In one study, breast cancer has been shown to be associated with herpesviruses, polyomaviruses, papillomaviruses and retroviruses [196]. TNBC samples (n = 100) along with matched (n = 17), and non-matched controls (n = 20) were screened using a microarray-based approach containing probe sets for parallel DNA and RNA detection of viruses and other human pathogenic microorganisms [197]. This PathoChip screening technology allowed detection of viral and bacterial signatures in the TNBC samples with significant association with the cancer samples compared to the non-matched and matched control samples [197]. Viral signatures belonging to Herpesviridae, Retroviridae, Parapoxviridae, Polyomaviridae and Papillomaviridae families were detected. Hepadnaviruses and Flaviviruses had the highest prevalence whereas Herpesvirus probes had the highest hybridization signal across the tumors [197]. TNBC samples fell into hierarchical groups showing at least two distinct microbial signatures; one hierarchical group was prevalent in viruses: a herpesvirus-signature (primarily β- and γ-herpesvirus-like); and a parapoxvirus signature (parapox virus family-like); flavivirus (hepatitis C and GB-like); polyomavirus (JC-MCPV- and SV40-like); retrovirus (MMTV-, HERV-K-, HTLV-like); hepadnavirus (hepatitis B-like) and papillomavirus (HPV-2, 6b and 18-like) [197]. Bacterial signatures could be found equally between the two hierarchical groups and bacterial probes included representatives of a number of families, some of which have been associated with cancers.

Due to the asymptomatic nature of early stage ovarian cancer, most patients are diagnosed at an advanced stage [198]. Identifying specific biomarkers for early diagnosis is paramount and can also can aid in risk assessment and prognosis. Using formalin-fixed paraffin-embedded ovarian cancer samples, matched and unmatched control samples, an ovarian cancer microbial signature was characterized using a DNA microarray approach and next generation sequencing for validation. Two predominant bacterial phyla were significantly associated with the ovarian cancer samples, Proteobacteria (52%) followed by Firmicutes (22%) and was distinct from the controls [199]. *Shewanella* signatures were detected with the highest prevalence in 91% of the cancers. This microbial signature associated with epithelial ovarian cancer is the first report linking specific phyla directly associated with the tumor and/or tumor microenvironment, whether the composition of the gut microbiota is similar to these results remains to be addressed. The same study also characterized the virome of these ovarian cancer patients. Among the signatures for viral families detected, 23% were identified as tumorigenic viruses and were prevalent in more than 50% of the cancer samples screened. Signatures of Retroviridae gave the highest signal followed by Hepadnaviridae, Papillomaviridae, Flaviviridae, Polyomaviridae and Herpesviridae [199]. Interestingly, HPV, HSV and other viral genomic integrations were detected in the ovarian tumor chromosomes, the highest number of viral integration sites were detected in human chromosomes for HPV16 with over 30 integrations, followed by HHV6a, HHV7 and HHV3 with less than ten integrations. Other viral integrations were detected from retrovirus, hepadnavirus, yaba monkey tumor virus and frog virus 3 [199]. Therefore, bacterial and viral signatures were associated with the ovarian cancer samples, as well as integration of viral sequences, and suggests an infectious and therefore an inflammatory component associated with the ovarian cancer samples compared to non-cancerous tissue.

Fungal signatures were also detected in ovarian cancer samples and included Aspergillus, Candida, Rhizomucor, Cladosporium fungus with the highest signal intensity detected with the probes for Cladosporium in all the cancer samples. Parasites associated with the ovarian cancer samples included Dipylidium and Trichuris. With a larger sample and validation set, this comprehensive oncobiome study may one day aid in early diagnosis of ovarian cancer. In addition to potentially inducing ovarian cancer, these microbial and viral signatures may also influence progression of ovarian cancer. Thus, studying microbial and viral signature differences in ovarian cancer patients over time (longitudinally) will be informative. Whether or not these oncobiome signatures directly or indirectly contribute as direct drivers to ovarian cancer or simply persist as secondary bystanders should be investigated [199].

11.3.2 Bacterial Flagellin, a TLR5 Agonist, Impacts Ovarian Cancer Progression

In the absence of treatment, in a murine model of ovarian carcinoma, tumor growth is significantly delayed with administration of a cocktail of broad-spectrum antibiotics, suggesting that microbiota and/or its modulation of inflammation aids in

ovarian tumor progression [155]. It was found that gut bacteria-derived TLR5 signaling drives tumor growth by suppressing endogenous antitumor immune responses. It was found that TLR5-dependent gut bacteria drives tumor progression at extra-mucosal locations by increasing systemic IL-6, which drives mobilization of myeloid-derived suppressor cells (MDSC) causing γδ T lymphocytes in TLR5-responsive tumors to secrete immunosuppressive galectin-1, which dampens antitumor immunity and accelerates tumor progression [155]. Thus, the gut microbiota, in a TLR5 signaling-dependent manner, systemically drives the up-regulation of IL-6 in the serum of tumor-bearing mice, subsequently promoting MDSC mobilization. Therefore, microbiota-dependent TLR5-IL6-MDSC-γδ T cell axis suppresses immunity in favor of tumor progression recapitulating the importance of myeloid cells at the intersection between innate and adaptive responses for manipulating immune responses that are pro-tumorigenic [155].

Within the same study, in TLR5-unresponsive tumor-bearing mice, IL-17 is consistently up-regulated, but only accelerates malignant progression in IL-6-unresponsive tumors [155]. Importantly, a cocktail of oral antibiotics abrogated differences in systemic IL-6 levels, mobilization of MDSCs and tumor growth when gut bacteria were eliminated between these tumor-challenged TLR5 WT and TLR5-deficient mice with significantly delayed tumor progression in WT mice. Cumulatively, this data supports the concept that flagellated bacteria and hematopoietic TLR5 **positive** cells at mucosal surfaces are driving differential tumor progression because: (1) reconstitution of TLR5 **positive** mice with TLR5-deficient (but not TLR5 **positive**) bone marrow recapitulated the delayed progression of syngeneic and spontaneous tumors observed in TLR5-deficient mice and (2) depletion of commensal bacteria with a cocktail of antibiotics abrogates any TLR5-dependent differences in tumor growth [152].

At least 23% of individuals in the general population are carriers of functional polymorphisms in *TLR* genes [200]. One of the most frequent polymorphisms is found in *TLR5*. Approximately 7.5% of the general population harbors a single dominant nucleotide polymorphism in *TLR5* (*TLR5*R392X) [201, 202] resulting in abrogated signaling in TLR5 (flagellin); heterozygous carriers have an enhanced susceptibility to Legionnaires' disease [201]. Contrasting differences in inflammatory cytokines and tumor growth are recapitulated in TLR5-responsive/unresponsive ovarian cancer patients. Myeloid leukocytes sorted from freshly dissociated human ovarian tumors from *TLR5*R392X heterozygous carriers showed lack of induction of IL-8 transcript levels in response to flagellin, compared to the same cell population sorted from patients with homozygous for TLR5. These results corroborate previous reports demonstrating that *TLR5*R392X carriers are functionally unable to respond to bacterial flagellin [155].

IL-17A transcript levels in ovarian carcinoma specimens were significantly higher in *TLR5*R392X carriers, compared to control patients homozygous for TLR5. Both γδ and αβ T cells contributed to IL-17 production in ovarian tumors. However, significant differences in IL-6 transcript levels were only observed between TLR5-responsive and nonresponsive ovarian tumor specimens. These data further support that in hosts where TLR5-dependent IL-6 does not dominate systemic tumor-promoting inflammatory responses through dramatic systemic up-regulation,

tumors grow faster in the presence of IL-17 overexpression, which is higher in the absence of TLR5 signaling [155].

To further investigate the link between IL-6 up-regulation and accelerated tumor progression in the presence of TLR5 signaling, the Cancer Genome Atlas dataset specific for ovarian cancer was analyzed. The proportion of long-term survivors (≥6 years after the ovarian cancer diagnosis) was significantly higher among *TLR5*R392X carriers, but not carriers of other non-functional polymorphisms, suggesting that, similar to the murine ovarian tumor model, TLR5 signaling drives accelerated malignant progression in ovarian cancer [155]. This finding warrants further investigation into mechanisms as well as the functional role the microbiota play in ovarian cancer progression.

Another unexpected outcome of the aforementioned study is a previously unrecognized contribution of immunosuppressive γδ T cells, which are dependent upon the interactions of TLR5+ immune cells with the microbiota. Relatively abundant in solid ovarian cancers, γδ T cells typically represent >6% of total leukocytes and outnumbering Foxp3+ Treg [155]. Although γδ T cells are more abundant at mucosal locations, impact by the microbiota on γδ T cells was not unexpected. What was surprising was the acquisition of regulatory attributes by γδ T cells appears to take place at extra-mucosal locations and not locally at places of direct interactions with the microbiota. Immunosuppressive activity of γδT cells is entirely dependent upon TLR5 signaling, in that γδ T cells in TLR5-deficient tumor-bearing mice paradoxically show protective activity [152]. Future studies will elucidate the plasticity of γδ T cells and the contribution of the gut microbiota required for the induction of immunostimulatory or immunosuppressive functions [203].

Several studies have confirmed a prominent role for the immune system in shaping the progress of ovarian cancer. ID8 ovarian cell line engineered to express the chemokine CCL28 (ID8-CCL28) is a more aggressive variant described by our group with ascites development correlating with tumor progression [204]. C57BL/6 animals acquired from JAX and HAR vendors were challenged with ID8-CCL28. Ascites development was delayed in JAX mice compared to HAR mice (A.F., unpublished data). Animals were treated with antibiotics that target gram-positive bacteria (vancomycin), gram-negative bacteria (neomycin, ampicillin and metronidazole). In mice administered vancomycin (primarily targets the gut with little systemic effects), the disparity in the survival between mice from different vendors was abolished; survival was similar between HAR mice receiving vancomycin and JAX mice (A.F., unpublished data). Gram-negative antibiotic treatment had no effect. Reconstitution of gut microbiota in vancomycin-treated JAX animals with gut bacteria from untreated HAR donors developed ascites tumors more quickly (lower survival rates) than the group that was reconstituted with JAX-derived bacteria. Overall, this finding shows that the ovarian tumor progression is gut microbiome-dependent and is transferable.

Ascites from vancomycin-treated ID8-CCL28 mice showed a decrease in both Th17 and MDSC cells, cell types that promote the progression of ovarian tumor progression and reduce the survival time of mice with tumors (A.F., unpublished data). Animals that received anti-TNFα antibody lived longer than the control HAR

group and their survival was similar to that of (low IL-17-cell-producing) JAX control mice, implicating TNFα in tumor progression. MDSCs and TH17 cells in peritoneal washes of animals receiving anti-TNFα antibody reduces these immunosuppressive populations. Similarly, blockade of TNFα reduced the same populations in ascites of tumor-challenged animals (A.F., unpublished data). These results demonstrate the gut microbiota regulates the function of peritoneal cavity-derived MDSCs and TNFα-induced IL-17-producing T helper cells.

11.3.3 Metagenomics and Ovarian Cancer

The metagenome is the sum of all genes and genetic elements and their modifications in the somatic and germ cells of a host plus all genes and genetic elements in all microorganisms (Bacteria, Archaea, Eukarya, and viruses) that live on or in that host at a given time. The metagenome has transient elements (e.g., during infection with a pathogen) and more persistent elements (e.g., infection with latent eukaryotic virus; presence of commensal bacteria) [205]. The microbiome is a complex community of microorganisms that infect humans and live in our tissues, contributes the majority of genetic information to our metagenome and, consequently, influences our resistance and susceptibility to diseases [205]. It is estimated that, in addition to integrated chromosomal viruses, each individual healthy human harbors more than ten permanent chronic eukaryotic viral infections that drive continuous activation of the immune system [77].

In addition to host factors (genetics and immunity), the gut microbiota and the metagenome (bacteria, virus, fungi) as a whole likely impacts, to one degree or another, cancer initiation and/or progression, and needs to be integrated into research paradigms for better understanding of the environmental factors that may play a role in ovarian cancer etiology and progression (Fig. 11.1). Interdisciplinary collaboration between pathology, bioinformatics, and computational biology using technologies (genomics, epigenomics, transcriptomics, proteomics, metagenomics) can lead to better understanding of etiologic heterogeneity and the impact of metabiome on disease evolution [206]. Cancer metagenomics is in its infancy, and in particular ovarian cancer, but may yield insights into biomarker discovery for diagnostic and prognostic tools.

We began detailing how ovarian cancer is an umbrella term for several histologically distinct types and the relative lack of effective biomarkers for screening, diagnosis, prognosis or treatment outcomes. It has been argued that a diagnosed "disease" is an imprecise phenotype. It is not because patients have been misdiagnosed however, there are many pathways to the same diagnosis. A diagnosis may be "clinically" precise but "mechanistically" imprecise [205]. Thus, clinical diagnoses are poor phenotypes for genetic studies unless a single mechanism is responsible for the diagnosis, as in the case of a rare gene mutation in a monogenic disease. The complexity of genome wide association study results is consistent with the existence of multiple disease subtypes within type 1 diabetes, IBD (ulcerative colitis or

Crohn's disease), each based on a specific mechanism. Support for this idea comes from the observation that subsets of IBD patients respond differentially to mechanistically distinct interventions [207]. So as ovarian cancer is further classified by histology and genetic makeup, understanding the impact of the metagenome on ovarian cancer initiation and progression should be addressed. New biomarkers in the form of microbial or viral signatures for different ovarian cancer histologies may offer new tools for screening or diagnosis, or may have prognostic value as well as predict treatment outcomes.

11.4 Conclusions

Understanding cancer-associated dysbiosis and in particular, dysbiota in ovarian cancer patients is in its infancy [208]. Understanding mechanistic details of the role of the microbiota in ovarian cancer progression may aid in modulating the microbiota with the use of pre and probiotics as well as potentially the optimal treatment combinations of chemoimmunotherapy with the use of antibiotics. Ovarian cancer progression is maintained in an inflammatory milieu, with a cytokine-rich ascites tumor microenvironment. Targeting cytokines in ameliorating symptoms in cancer may ameliorate inflammation but at the same time, may also abrogate host defense against infections in an otherwise immunocompromised patient. Novel classes of therapies are needed that target upstream pathways that are disease-modifying rather than symptom-based. Understanding the role of the microbiota in ovarian cancer progression may expand the armamentarium against standard of care ovarian cancer treatments and those under investigation, particularly novel immunotherapies and combinations such as chemoimmunotherapy and radioimmunotherapy.

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Chapter 12
Hepatocellular Cancer Induced by Infection

David E. Kaplan, Kyong-Mi Chang, and Arun Sanyal

Abstract Hepatocellular carcinoma is the fifth most common cancer and second leading cause of cancer death worldwide. Chronic viral infections contribute to approximately three-fourths of these cancers either as direct carcinogens or indirectly mediated through progressive hepatic fibrosis and cirrhosis. Bacteria, specifically the gut microbiome, also contributes to the in the genesis of hepatocellular carcinoma. Obesity-related nonalcoholic fatty liver disease and alcoholic liver disease, the major non-viral causes of chronic liver disease predisposing to liver cancer, alter the composition of the gut microbiome, which appears to foster development and progression of pre-malignant and malignant liver neoplasms. Emerging data implicates patterns of dysbiosis with alterations of bile acid metabolism, insulin resistance, fibrogenesis, and gut barrier integrity that contribute to intrahepatic inflammatory signaling and carcinogenesis. In vitro, small animal model, and human data supporting the role of chronic viral infection and bacterial derangements in hepatocarcinogenesis will be reviewed.

Keywords Hepatocellular carcinoma · Cirrhosis · Microbiome · Hepatitis B · Hepatitis C · Alcohol · Toll-like receptors · Bacterial translocation · Bile acids

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12.1 I. Introduction

Historically, hepatocellular carcinoma (HCC) has been largely considered in the context of infectious diseases due to its inextricable linkage with chronic hepatitis viral infections. Of the estimated 782,000 incident annual cases [1], approximately 70% of cases are attributed to underlying chronic viral hepatitis B or C [2]. Recently, there is growing interest not only in viruses, but also bacteria, specifically the gut microbiome, in the genesis of HCC. Obesity, nonalcoholic fatty liver disease, and alcohol-related liver disease, the major non-viral causes of HCC are associated with significant dysbiosis. Emerging data suggest that the composition of the gut microbiome both impacts and is impacted by chronic liver disease, and this interaction appears to be relevant both in the development and progression of pre-malignant and malignant liver diseases. Specific bacterial species in the intestines may alter bile acid metabolism, insulin resistance, fibrogenesis, and gut barrier integrity leading to alterations in intrahepatic inflammatory signaling ultimately potentiating cancer growth. While several rodent models have elucidated the possible interaction of the gut microbiome and human hepatocellular carcinoma, to date a paucity of data exist to confirm the relevance of these findings to human chronic liver disease. In this review, we will review the role of the microbiome in the development of pre-malignant liver disease and the data supporting the importance of infectious processes on liver cancer development (Table 12.1).

Table 12.1 Mechanisms of infection-driven hepatocellular carcinoma

Mechanism	Example
Insertional mutagenesis	Integration of HBV genome into host chromosomal DNA during high level replication [10]
Transcriptional activation	HBV x protein transcriptional activation of chromatin remodeling, autophagy and miRNA [13]
	HBV pre-S2 induction of bcl2 [10–13] HCV Core activation of wnt/β-catenin pathway
Reactive oxygen species/ER stress	HBV pre-S mutants, HCV Core protein, HCV NS3 protein
Epigenetic alterations	HCV epigenetic alteration of SFRP, FOXA1, FOXA2, HNF4A, CDKN2A, ApoE [21] HBV epigenetic alteration of RARβ2, IGJBP-3 [21]
Inflammation/ immunosuppressive microenvironment	Induction of antiviral innate and adaptive immune responses including regulatory responses such as regulatory T-cells, myeloid dendritic suppressor cells, fas and program-death ligand expression [27–31]
Cirrhosis-related changes	Telomerase reactivation, suppression of tumor suppressor genes, p53 mutations [32]
Dysbiosis-related hepatic inflammation	Lipopolysaccharide activation of TLR4 [64] Deoxycholic acid induction of FXR [60]

12.2 Infections and the Epidemiology of HCC

Primary HCC is the fifth most common cancer and second leading cause of cancer death worldwide, killing over 745,000 individuals annually [1]. The largest burden of liver cancer occurs in Southeast Asia and Northern and Western Africa, predominantly due to endemic chronic hepatitis B virus (HBV) infection. For example, in a Taiwanese study of 22,707 men, chronic hepatitis B surface antigen (HBsAg) carrier state was associated with increased HCC risk by over 200-fold and increased liver-related mortality [3]. Aflatoxin B1, a fungus-derived toxin produced by *Aspergillus flavus* and *A. parasiticus* that contaminates peanuts and other grain stores in hot, humid climates, is a frequent co-factor contributing to HCC development in these regions [4]. In Japan, North America and Europe, the most common etiologies of liver disease predisposing to HCC are chronic hepatitis C virus (HCV) infection, alcoholic cirrhosis, other causes of cirrhosis, and increasingly non-alcoholic fatty liver disease (NAFLD). Approximately 80% of liver cancers emerge in the setting of pre-existing liver cirrhosis with the majority of the remainder arising in patients with intermediate-to-advanced fibrosis and rarely occurring in histologically normal livers [5]. There have been recent case reports implicating NAFLD in cases of non-cirrhotic HCC [6], and some non-cirrhotic HCV patients rarely develop HCC as well. Nonetheless, most HBV-related HCC and the vast majority of HCC from other etiologies such as hepatitis C arise in the setting of liver cirrhosis.

12.3 Clinical Aspects of HCC

Hepatocellular carcinoma is a highly lethal cancer, with annual incidence and mortality rates that are nearly identical [1]. The two primary reasons for poor survival rates include (1) a high frequency of diagnosis at intermediate to advanced stages due to the rarity of clinical symptoms in earlier stage disease; and (2) a high frequency of associated liver dysfunction that not only adds a competing risk for mortality but which also may limit cancer-directed treatment options [7]. Selected early stage HCC may be cured by surgery such as liver resection or liver transplantation or by ablative techniques such as microwave ablation, radiofrequency ablation, cryoablation, chemical ablation, and/or stereotactic beam radiotherapy. Intermediate and advanced stage liver cancer may be palliated by locoregional transarterial therapies such as transarterial chemoembolization or radioembolization, systemic therapies such as sorafenib and regorafenib (in addition to many other candidates in clinical development), and radiotherapy. Five-year survival rates for hepatocellular carcinoma remain <20% [7, 8] with a median survival of <10 months in the United States [7, 9].

12.4 Infections and the Pathogenesis of HCC

12.4.1 Chronic Viral Hepatitis B and C

Chronic viral infections of the liver, specifically HBV and HCV, can potentially impact the genesis of HCC both by the direct actions of viral proteins and products derived from active viral replication and from the host responses generated due to the viral infection.

12.4.1.1 Viral Factors

Several HBV viral gene products (e.g. HBV envelope, HBV X, HCV core, HCV NS3, HCV NS5) may promote hepatocyte-transformation through interactions with cellular factors and increasing endoplasmic reticulum (ER) stress with unfolded protein responses [10]. HBV preS/S and X proteins can act as transcriptional activators, and contribute to pro-oncogenic transcriptional program [10–13]. Furthermore, hepatic expression of viral gene products (e.g. large HBV envelope or HCV NS3) have been shown to mediate liver cancer in animal models [14, 15]. As a DNA virus with an RNA intermediate that is reverse-transcribed, HBV can also integrate into the host genome, with the potential to activate cellular oncogenes or disrupt proliferation checkpoints. HBV DNA integration (as well as clonal hepatocyte proliferation) may occur early in chronic hepatitis B [16], but in a random fashion (unlike woodchuck hepatitis virus in which HCC is associated with the activation of myc family proto-oncogenes by the insertion of viral enhancer sequence) [17, 18]. Nevertheless, HBV DNA integration can result in chimeric HBV fusion transcripts that regulate microRNA activity and epigenetically promote HCC development [19–21]. As a cytoplasmic RNA virus, HCV does not integrate into host genome. However, overexpression of HCV core protein [15], Core-E1-E2 [22], full-length virus [23, 24], NS3 [25] and NS5 protein [26] might accelerate inflammation-associated or toxin-induced carcinogenesis possibly by generating steatogenic reactive oxygen species or by altering miRNA expression.

12.4.1.2 Host Factors in Viral Hepatitis Related HCC

For both viruses, ineffective host immune response to persistently virus-infected liver can lead to chronic inflammation and hepatocellular injury with increased cell turnover, oxidative stress with metabolic alterations, DNA damage, cellular senescence, and telomerase reactivation as well as the induction of multiple immune regulatory pathways that may further dampen antiviral immunity and tumor surveillance [10, 27–31]. Chronic viral hepatitis can progress to cirrhosis, a well-known risk factor for HCC with its associated procarcinogenic microenvironment. The specific mechanisms by which cirrhosis drives hepatocarcinogenesis still remain

incompletely characterized. However, certain pre-malignant changes such as telomerase activation, cellular senescence, epigenetic suppression of tumor suppressor genes such as RASSF1A, and mutations in oncogenes such as p53 (reviewed in Ramakrishna et al. [32]) can precede the development of malignancy in cirrhotic nodules and are believed to contribute.

12.4.2 The Intestinal Microbiome and Hepatocellular Carcinoma

Data from several mouse models of chronic liver disease that develop hepatocellular carcinoma have established a strong association between the composition of the gut microbiome with liver cancer development and/or progression. The majority of these data have been developed in genotoxic rodent models in which the carcinogenicity of a known direct mutagen is modulated by the enteric bacterial colonization. For instance, the carcinogenicity of aflatoxin B1 in C3H/HeN mice was shown to be strongly modified by the enteric colonization of *Helicobacter hepaticus* [33]. In this study, *H. hepaticus* colonization induces hepatic expression of NFκB without localizing to the liver, likely due to portal circulation of TLR ligands, suggesting that permissiveness to genotoxic hepatocellular carcinomas is fostered by bacteria-induced hepatic inflammation [33]. Of interest, tumors that formed in this model expressed β-catenin, suggesting wnt-activation might be a mechanism by which *H. hepaticus* could alter tumor formation [33]. Similarly, the oncogenicity of intrahepatic overexpression of hepatitis C transgenes appears to be regulated by the presence or absence of inflammatory bacterial colonization of the intestine [33].

In other models, the microbiome is suggested to play a greater role in fostering tumor growth once initiated, rather than in driving initial tumorigenesis. The growth of previously transformed Hepa1-6 murine hepatocellular carcinoma cells allografted on C57Bl/6 mice, for instance, could be reduced by 40% with co-administration of probiotics (*Lactobacillus rhamnosus* GG, VSL #3, and *E. coli* Nissle 1917) [34]. Dapito et al. [35] utilizing a combination carcinogen/fibrogenesis model with neonatal diethylnitrosamine (DENA) administered prior to carbon tetrachloride (CCl_4), found that germ free wild-type mice developed similar numbers but smaller tumor sizes than conventional wild-type mice. In this model, gut sterilization reduced the size but not number of tumors, particularly if administered after the first 4.5 months after neonatal DENA exposure. Furthermore, exogenous lipopolysaccharide (LPS) also drove tumor growth through upregulation of hepatic hepatocyte growth factor and hepatic stellate cell expression of epiregulin, a protein that is known to be increased in livers of alcoholic liver disease patients [36]. Epiregulin and TLR4 knockout mice subjected to a similar cancer induction protocol had a partially reduced tumor size but not number, suggesting that epiregulin may also be one of several factors that promotes tumor progression independent of tumor initiation. TLR4 expression, particularly on myeloid cells within the liver, has also been strongly implicated in the progression of DENA-induced HCC in rats, possibly

mediated by intrahepatic STAT-3 phosphorylation [37, 38]. These models suggest that the hepatic inflammatory response to translocated products of enteric gram-negative rods provides critical trophic support to limit apoptosis of genotoxically-transformed hepatocytes, fostering growth but not altering tumor initiation.

Some evidence suggests that DENA not only acts as a genotoxin, but itself impacts gut microbial diversity. In Sprague-Dawley rats, DENA resulted in dysbiotic changes to the microbiome, with increased representation of bifidobacterium and enterococcus associated [38]. Probiotic or antibiotic administration [37] attenuated this dysbiosis, resulting in reduced HCC number and growth suggesting a complex interaction between the genotoxic agent, the gut and the liver.

While these models do not authentically recapitulate human chronic liver disease predisposing to hepatocellular carcinoma, the models do suggest that alteration of the microbiome are critical drivers of hepatocarcinogenesis. Human observational studies suggest the plausibility of this model of pathogenesis, offering some hope that interventions that interfere with these processes might be clinically useful for chemoprevention.

12.4.3 Role of the Host-Microbial Interactions in the Development of Liver Fibrosis

The intestinal microbiome may modulate the development of HCC both directly and indirectly by modulating metabolism, inflammation and cell stress within the liver. Microbiota may translocate from the intestine to other parts of the body or may change the intestinal barrier function thereby allow ingress of bacterial molecules in to the body where they can affect the redox state, induce inflammation and alter metabolism and potentially even activate carcinogenic pathways. Bacteria also metabolize dietary constituents and endogenous substances particularly bile acids to produce bacterially modified molecules that can affect oncogenesis.

12.4.3.1 Endotoxemia and Chronic Hepatic Inflammation

As noted above, the majority of human hepatocellular carcinomas arise in the background of hepatic fibrosis and cirrhosis. It has long been appreciated that both the underlying causes of hepatic fibrosis, such as chronic alcohol exposure, and the consequences of cirrhosis such as portal hypertension are associated with increased portal and systemic exposure to bacterial products such as endotoxin and CpG-methylated bacterial DNA. For instance, Bode et al. in the 1970s demonstrated that elevated serum levels of endotoxin could be found in two-thirds of alcoholic cirrhotics, nearly half of non-alcoholic cirrhotics, as well as nearly half of non-cirrhotic individuals acutely exposed to large quantities of alcoholic beverages [39]. Recently increased systemic endotoxemia has been observed in obesity, type 2 diabetes and NAFLD all conditions associated with an increased cancer risk [40, 41].

12.4.3.2 Bacterial Translocation

Rat models of alcoholic cirrhosis confirm that alcohol exposure promotes the translocation of enteric bacteria to mesenteric lymph nodes [42]. Altering the gut microbiome in rat models of alcoholic cirrhosis either by antimicrobial decontamination or by supplementation with specific probiotic bacterial species such as *Lactobacillus rhamnosus* GG (LGG) significantly attenuates circulating bacterial endotoxin levels, which in turn is associated with reduced steatohepatitis and fibrogenesis, strongly implicating the translocation of specific bacterial products in the pathogenesis of liver injury [43, 44]. However it is yet unclear if bacterial translocation itself is involved in the genesis of HCC.

12.4.3.3 Altered Gut Barrier Function

Some data suggest that ethanol itself fosters gut microbial translocation and endotoxin penetration into the portal circulation by directly increasing intestinal permeability. *In vitro*, ethanol disrupts intestinal epithelial tight junctions by altering the cellular distribution of key tight junction proteins, zonulin-1 and occludin [45, 46]. Changes in tight junction integrity have also been identified in non-alcoholic fatty liver disease, associated with increases of alcohol-producing Escherichia species, reduced expression of occludin, and increased intestinal inflammatory cytokine expression [47]. Excess of alcohol-producing Escherichia in the gut microbiome has also been described by some but not all studies of hepatitis C-related cirrhosis [48–50]. Other data, however, suggest a more indirect effect of alcohol mediated by alteration of the composition of gut microbiome itself (e.g. expansion of gram-negative Proteobacteria species), fostering microbially-mediated changes in tight junction permeability, that can be attenuated by probiotics such as LGG *in vivo* [44, 51].

Progression of liver disease itself, even in the absence of alcohol exposure, also increases gut permeability. For instance, Choi et al. showed that intestinal permeability in humans as measured by urinary excretion of orally administered polyethylene glycol progressively increases in patients with worsening viral liver disease and correlates strongly with plasma endotoxin levels [52]. Increased intestinal permeability in advancing cirrhosis has been linked to reduced expression of tight junction proteins such as occludin and claudin-1 in intestinal villi [53], possibly due to TNFα-mediated induction of miR122a [54], or IL-6 mediated upregulation of claudin-2 [55]. Increased gut permeability in cirrhosis with immune dysregulation was also suggested by systemic antibody response to commensal bacteria generally contained in the gut by the innate lymphoid cells [56]. Human clinical trial data confirm that the probiotic LGG administered to cirrhotic patients reduces circulating endotoxin levels [57] thought due to improvement of intestinal tight junction integrity due to alteration of the microbial repertoire [58].

12.4.3.4 Bile Acids

Similar but distinct findings have been found in the murine choline-deficient high-fat diet model (MCDHFD) of nonalcoholic steatohepatitis. In this model, hepatic injury can be exacerbated by disrupting gut barrier functions using the detergent dextran sulfate sodium (DSS), resulting in greater fibrosis and HCC development. MCDHFD however also promotes overgrowth of Clostridial cluster XI species [59]. Clostridial cluster XI species are of specific interest because they are among a small number of bacterial species that express 7α-hydroxylases that can convert primary bile acids to secondary bile acids. Further evidence that alteration of Clostridial cluster IX gram-positive species in the gut can promote hepatocarcinogenesis comes from the dimethylbenz(a)anthracene (DMBA) murine model in which DMBA, an inducer of ras mutations, will result in hepatocellular carcinoma when only administered with a high-fat diet. Using mice with a reporter for a senescence-related gene $p21^{Waf1/Cip1}$, Yoshimoto et al. found that antimicrobial decontamination of the gut in these mice reduced HCC formation but not via reduction of gram-negative bacteria-derived LPS but via alteration of 7α-dehydroxylation of primary bile acids by gram-positive Clostridial cluster IX species [60]. Inhibition of 7α-dehydroxylation with specific inhibitors or the hydrophilic bile acid ursodiol reduced deoxycholic acid levels, as well as hepatic stellate senescence and HCC development. Intrahepatic inflammatory cytokines such as TNFα, IL-6 and IL-1β, were more strongly increased by high fat diet, as was FXR-mediated insulin resistance, and co-associate with reduction of Lactobacillus species and marked increases in Clostridium cluster IX species. These murine data support a bile acid/microbiome model of hepatocarcinogenesis [61]. Since only specific microbes, specifically Eubacteria and Clostridial cluster IX and XIVa, produce 7α-hydroxylases that convert unconjugated primary bile acids into unconjugated secondary bile acids, overgrowth of these species could result in overproduction of deoxycholic acid in the intestines. Deoxycholic acid is toxic to bacterial membranes and when administered to mice itself results in dysbiosis. Deoxycholic acid is also a strong inducers of FXR and the vitamin D receptor, which drives hepatic inflammation and hepatic stellate cell senescence, factors strongly associated with HCC in mice models [60]. Intrahepatic T-cell activation may also be a key effect modifier in the MCDHFD, in which intrahepatic NKT cell activation fosters the accumulation of lipid, the NASH phenotype (ballooning, fibrosis), and HCC development [62].

12.4.3.5 Immune-Inflammatory Effects of the Intestinal Microbiome

Once into the portal system, various bacterial products exhibiting pathogen-associated molecular patterns (PAMPs, e.g. bacterial flagellins, endotoxin/lipopolysaccharide, CpG DNA, lipoteichoic acid) can interact with pattern recognition receptors (PRR) such as toll-like receptors (TLR) and nucleotide-binding

oligomerization domain-containing (NOD) proteins expressed by parenchymal (hepatocyte) and non-parenchymal liver cells (e.g. Kupffer cells, liver sinusoidal endothelial cells, and hepatic stellate cells) [63]. Of these interactions, increased portal circulation of endotoxin has been most specifically linked to enhanced hepatic fibrosis via activation of TLR4, particularly that which is expressed on hepatic stellate cells. The importance of endotoxin in promoting hepatic fibrosis was first suggested by Seki et al. in the murine bile duct ligation (BDL) model [64]. These investigators observed that compared to wild type mice, $tlr4^{-/-}$ mice manifested reduced hepatic fibrosis after BDL, and proceeded to show that endotoxin derived from enteric gram-negative rods sensitizes hepatic stellate cells to become activated by Kupffer-cell derived TGFβ to drive liver fibrogenesis. The role of TLR4 signalling in fibrogenesis has since been confirmed in other animal models [65, 66] and has been associated with the stage of fibrosis in humans with NASH [67, 68]. TLR4 may signal through two separate adaptors, MyD88 and TRIF, to activate NFκB or IRF3 respectively. MyD88 upregulation has been observed *in silico* in human NASH and ASH [69, 70]. Other bacterial or viral PAMPs signaling through TLR3 or TLR7 upstream of MyD88 have also been implicated in hepatic fibrosis [71] More recent animal data suggests that TLR4-expressing Kupffer cells responding to circulating LPS by producing TNFα may also indirectly contribute to hepatocyte injury in a TRIF-dependent manner [66].

Overall, data strongly suggest that disruption of gut microbial barrier due to chronic liver disease-induced dysbiosis contributes to the pathogenesis of liver injury, inflammation and fibrosis, which in turn fosters ongoing dysbiosis creating a feed forward loop. The subsequent development of hepatocellular carcinoma also appears to be strongly modulated by inflammatory signals mediated by the gut microbiome.

12.5 Summary and Conclusion

Hepatocellular carcinoma is inextricably linked to microbes, both in the genesis of the antecedent chronic hepatic inflammatory state and in the initiation and promotion of neoplasia. Viral infection of hepatocytes can be directly mutagenic but more frequently facilitates inflammation-associated carcinogenesis directly through complex interaction with host metabolic pathways or indirectly through innate and adaptive antiviral programs. Once these processes are initiated either by viral or hepatotoxic injuries, toxin- or dysbiosis-driven gut hyper-permeability and associated intestinal bile acid signaling appear to potentiate intrahepatic inflammatory signaling and subsequent carcinogenesis. Carefully performed human studies and novel animal models are needed to further unravel the complex interactions in which microbiome contributes to HCC development.

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Chapter 13
Manipulation of the Host Immune Response by Small DNA Tumor Viruses

Elizabeth A. White, Srinidhi Shanmugasundaram, and Jianxin You

Abstract Viral infection accounts for up to 15% of cancer cases worldwide. Many oncogenic viruses maintain asymptomatic, persistent infections in immunocompetent hosts and only induce tumorigenesis in the immunocompromised population, highlighting the critical role of the host immune system in controlling virus-induced carcinogenesis. Emerging evidence demonstrates important themes of immune evasion utilized by oncogenic viruses in order to maintain persistent infection. In this chapter, we focus on the immune evasion tactics employed by two small DNA tumor viruses: human papillomavirus (HPV) and the more recently discovered Merkel cell polyomavirus (MCPyV). We will highlight how their manipulation of host immune responses helps to create a cellular environment that supports persistent infection and viral oncogenesis. A comprehensive understanding of the immunomodulatory mechanisms utilized by these viruses during the onset of oncogenesis may contribute to the development of novel therapeutic strategies targeting virus-associated cancers.

Keywords Merkel cell polyomavirus · Human papillomavirus · Innate immunity · Adaptive immunity · Immune evasion · Immunomodulatory therapy

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13.1 Manipulation of Host Immunity by HPV and MCPyV

The human immune system is comprised of the innate and adaptive immune systems and the more recently identified intrinsic immune system. Together, this tripartite immune system recognizes invading pathogens and foreign stimuli and launches cellular responses to eliminate them; however, each branch of the immune system does so through distinct mechanisms at different time points following initial exposure [1, 2].

The innate immune system constitutes the initial, non-specific defense against a pathogen and therefore is able to act almost immediately following antigen entry into the body. This system is dependent on a set of germline-encoded pattern recognition receptors (PRRs) that are able to recognize a broad range of pathogen associated molecular patterns (PAMPs) in various extracellular and intracellular locations [3]. PRRs can sense and respond to motifs of common pathogens such as the unmethylated CpG motifs of DNA viruses and ultimately activate signaling cascades that alert immune cells to control infection [4]. Several toll-like receptors (TLRs) play a critical role in recognizing the presence of viral DNA extracellularly as well as in endosomal compartments while a broad repertoire of intracellular PRRs detects viral DNA within the cytosol [4, 5]. The recognition of motifs specific to viral DNA helps these receptors distinguish it from natural host DNA and mount an appropriate response to eliminate infection. Nevertheless, viruses are constantly evolving to develop more complex mechanisms in order to escape innate immune detection [6].

The adaptive immune system is induced to develop an antigen-specific response following exposure to a specific pathogen. After an encounter with a certain antigen, responding B- and T-lymphocytes clonally proliferate in a way that allows these cells and/or the antibodies they produce to recognize that antigen. A subset of these B- and T-lymphocytes also differentiate into memory cells that can activate a stronger, faster response upon subsequent encounters with the same antigen [2]. These lymphocytes activate specific signaling cascades that ultimately aim to eliminate infection through various humoral and cell-mediated mechanisms. What distinguishes the adaptive immune response from the innate immune response is its specificity to a particular antigen as well as the immunological memory established in this process. In comparison to the innate immune response, the adaptive immune response is more complex and, due to this element of specificity, slower to process and respond to antigen [3]. Many viruses have high mutation rates in their surface proteins due to processes known as antigenic shift and drift. This helps them to evade detection by the adaptive immune system and considerably complicates the development of prophylactic vaccines against these viruses [7].

The intrinsic immune system has long been thought of as a subdivision within innate immunity; however, several distinctions revealed in recent studies merit its consideration as a separate system. For example, whereas effectors of innate immunity recognize and respond to a broad range of pathogenic signaling, intrinsic immune activity does not require any virus-triggered signaling or intracellular

communication [1]. The effectors of intrinsic immunity are constitutively expressed, enabling this system to respond immediately following viral entry. Unlike either the adaptive or innate immune systems, the production of intrinsic immune effectors remains constant even after infection—consequently, this system can be overwhelmed and become ineffective at higher levels of viral load [1, 8].

Two oncogenic DNA viruses, Human Papillomavirus (HPV) and Merkel Cell Polyomavirus (MCPyV), are the focus of this chapter. The molecular patterns present in the viral particles themselves or generated during the infectious life cycle can trigger host immune responses leading to the clearance of infection. However, both viruses have the ability to maintain latent and persistent infection in infected individuals over time frames of years to decades. Clearly, these viruses have evolved strategies to escape host immune surveillance. Because persistent viral infection is critical for achieving virus-driven tumorigenesis, it is important to examine the molecular strategies employed by these oncogenic viruses to evade immune detection and establish chronic infections.

The chapter begins with an overview of the basic biology of these two viruses. Next, we highlight the importance of host immune control of HPV and MCPyV infection, which is illustrated by the increased severity of disease caused by both viruses in immunocompromised populations. The individual mechanisms by which HPV and MCPyV interact with the host adaptive, innate, and intrinsic immune systems are currently being investigated by many laboratories, and we will highlight key recent findings, therapeutic implications, and discuss outstanding questions in the field.

13.2 Biology of the Small DNA Tumor Viruses

13.2.1 Human Papillomavirus

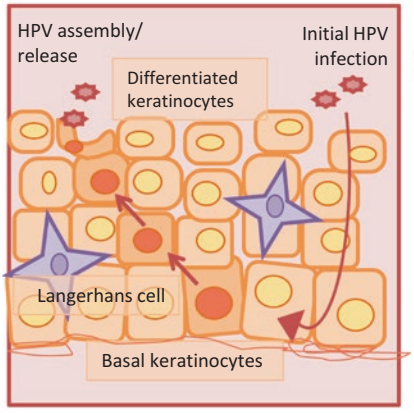
Transmissible cancers in birds were described in the early 1900s, but several decades passed before Richard Shope characterized the first such cancer in mammals [9–11]. Further study of the transmission of tumors between rabbits through cell-free extracts led to the identification of the first mammalian tumor virus: the Shope Papillomavirus, now termed cottontail rabbit papillomavirus (CRPV). Hundreds of additional papillomaviruses have since been identified. All of them have circular double-stranded DNA genomes of approximately 8 kbp, have a strict tropism for the mucosal or cutaneous stratified squamous epithelium, and are highly species-specific [12]. The more than 200 known human papillomaviruses (HPV) are phylogenetically classified into five genera (alpha, beta, gamma, mu, and nu) based upon the sequence of their L1 gene, which encodes the major capsid protein [13]. Fewer than 15 of the genus alpha viruses are the so-called ‘high-risk’ mucosal virus types that together are responsible for 5% of the worldwide cancer burden. These high-risk HPVs cause nearly all cervical cancer, some other anogenital cancers, and an increasing proportion of oropharyngeal cancer [14–16].

The arc of study of tumor viruses, including papillomaviruses, in the past century has been remarkable: from the initial identification of transmissible tumors to the recognition of Peyton Rous' work on sarcomas caused by filterable agents with a Nobel Prize in 1966 took almost 50 years. The work of Harald zur Hausen and colleagues in the 1980s that suggested a link between HPV infection and cervical cancer [17, 18] laid the foundation for the approval of safe, effective prophylactic HPV vaccines in 2006 and was recognized with a Nobel Prize in 2008. Today, new papillomavirus genotypes are frequently identified and the mechanisms by which the papillomavirus-encoded proteins manipulate the host cellular environment are studied intensely.

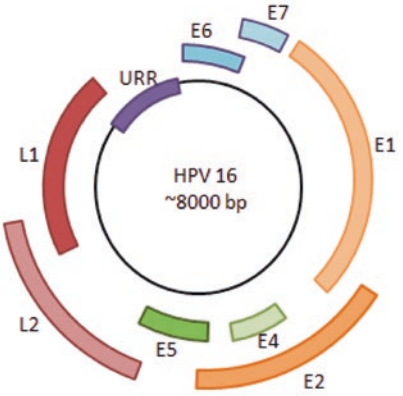
Although infection with human tumor viruses is common, disease is rare. This is true of both the high-risk, cancer-associated mucosal HPVs and the many cutaneous HPVs that are resident on the skin of healthy individuals. Globally, the prevalence of HPV infection is 11–12% in women with normal cervical cytology. Prevalence declines with age, and it is estimated that 80–90% of women are infected with HPV over their lifetimes [14, 15]. Cutaneous viruses are readily detectable and the majority of healthy individuals harbor beta-HPV DNA on a variety of anatomical sites [19–22]. The high frequency of infection emphasizes that these viruses are able to bypass detection by host immune responses and establish themselves in host cells. At the same time, the fact that the vast majority of these infections are cleared by the immune system and do not progress to cause cancer, or indeed cause any apparent lesion, demonstrates that HPV infections are well controlled by a healthy immune system.

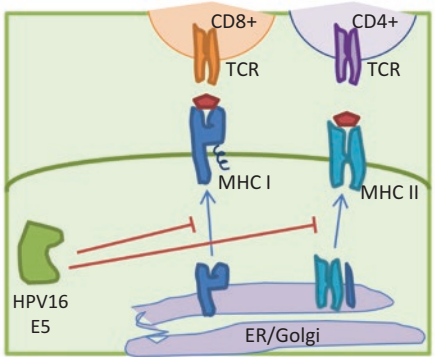
Replication of an HPV in the epithelium occurs slowly and concomitantly with the differentiation of the tissue [12] (Fig. 13.1). The virus establishes an initial infection in basal cells, where the DNA genome becomes established as a nuclear episome and the viral early genes are transcribed. Genomes are maintained in dividing cells and divide once per cell cycle along with the host chromosomes. After these cells exit the cell cycle and begin their differentiation program the later stages of the viral life cycle, including replication of the viral DNA genome to a high copy number, can occur. The capsid proteins encoded by the HPV late genes are produced in terminally differentiated cells and viral particles are released from desquamating cells.

This differentiation-dependent life cycle is frequently proposed to help papillomaviruses evade immune detection [13, 23]. Infected basal keratinocytes maintain low levels of viral gene expression and link viral DNA replication to host cellular genome replication, minimizing immune stimuli. Higher levels of viral gene expression that bring the potential to trigger a robust immune response are restricted to the upper layers of the epithelium where there is less immune surveillance. However, the relationship between HPV replication and immune evasion is more complicated than this model would suggest. There is considerable immune surveillance in the epithelium and the HPV early proteins that are expressed throughout the viral life cycle suppress immune signaling using several different mechanisms. The viruses are adept at limiting inflammation in the infected tissue and helping to prevent their own detection and clearance by the immune system. This chapter will highlight



The synchronization of the viral life cycle with keratinocyte differentiation avoids the induction of viremia and cytolysis, thus circumventing the activation of inflammatory responses and resident immune cells.





HPV infection reduces the presentation of antigens by MHC molecules.

Fig. 13.1 HPV immune evasion in the differentiating epithelium. The HPV life cycle is linked to the differentiation program of the stratified squamous epithelium. T cells and Langerhans cells in the epithelium detect infected cells and drive low levels of viral gene expression. Several viral mechanisms of immune evasion act to counter the host immune response, including MHC down-regulation by HPV16 E7

some of the well-established and emerging mechanisms by which this virus-host balance is achieved.

In a rare subset of high-risk HPVs infections, normal control of the virus replication cycle is lost, initiating a progression of events that leads to dysregulation of the cell cycle and ultimately to cancer. Transformation of a host cell is an evolutionary dead end for HPVs and other tumor viruses, and an HPV-transformed cell no longer produces infectious virus. The high-risk HPV E6 and E7 oncoproteins drive the progression to cancer [24]. High risk HPV E6 and E7 proteins work together to immortalize and/or transform cells *in vitro*, and a longstanding model holds that HPV E7 proteins bind to and inactivate the retinoblastoma tumor suppressor (pRb)

and related pocket proteins, allowing progression through the cell cycle in otherwise terminally differentiated and non-cycling epithelial cells. This enables the production of cellular machinery necessary for replication of the viral DNA genomes, but the resulting unscheduled DNA replication triggers stress signals and causes a stabilization of the tumor suppressor p53. Consequently, high-risk HPV E6 proteins recruit p53 and the ubiquitin ligase E6AP to form a complex that allows the ubiquitination and proteasome-mediated degradation of p53. More recent updates to this model have been proposed [25] and additional oncogenic events are likely to be involved. It has long been thought that the main way in which regulatory control of E6/E7 is lost is through integration of the HPV genome into the cellular DNA and disruption of the gene encoding the HPV E2 transcriptional regulator, although more recent studies suggest that dysregulation of E6/E7 expression might be achieved in one of several ways [26]. In any event, the oncogenic activities of E6 and E7 continue to be studied extensively and it is important to remember that the behavior of an HPV-infected cell in which virus replication is occurring may be quite different than that of an HPV-positive cancer cell.

E6 and E7 are expressed throughout the viral life cycle and maintain an environment in the terminally differentiated cell in which the cellular machinery necessary for replication of the viral DNA genome is available. The limited coding capacity of HPVs means that their proteins are multifunctional, and several HPV proteins including E6 and E7 have also been recognized for some time to modulate immune signaling. It has been proposed that there is overlap between the pathways that control the cell cycle and those that detect pathogens via an innate immune response, and that tumor viruses both transform cells and evade immune detection because they target cellular molecules and pathways that are at the interface of these signaling pathways [27].

In the laboratory, the differentiation-dependent HPV replication cycle and the viruses' species specificity present experimental challenges. Many of the experiments described in this chapter have been performed in epithelial cells that harbor episomal or integrated HPV genomes, or a mix of the two; in cells that produce one or more of the HPV proteins from heterologous expression vectors; or using HPV pseudovirions: viral particles produced by transfection of the HPV capsid genes plus a reporter plasmid into a packaging cell line. Other experiments were conducted using fully transformed HPV-positive cancer cell lines. Technological advances and the discovery of new animal viruses that can be studied in model systems are improving the ability to study immune modulation by papillomaviruses, but there remains much to learn about how these viruses co-exist with their natural hosts.

13.2.2 Merkel Cell Polyomavirus

Merkel cell polyomavirus (MCPyV or MCV) is a new member of the Polyomaviridae family discovered in 2008 in Merkel cell carcinoma (MCC), a neuroendocrine carcinoma of the skin [28–30]. Despite getting its name from the cancer in which it was

discovered, MCPyV is present widely in the general population [31, 32]. The infection is asymptomatic in healthy humans but can lead to the MCC skin cancer in elderly and immunocompromised individuals such as AIDS patients and organ transplant recipients. Besides immune suppression, advanced age and excessive exposure to sunlight and ultraviolet (UV) radiation are the other major risk factors for MCPyV-associated MCC.

MCC was first described by Cyril Toker in 1972 as a poorly differentiated trabecular tumor of the dermis [33]. It has turned out to be one of the most aggressive skin cancers with a 5-year survival rate less than 45% and a mortality rate of almost 33%—higher than that for melanoma [34–36]. Furthermore, MCC is responsible for more deaths than some more well-known cancers such as cutaneous T-cell lymphoma and chronic myelogenous leukemia [36, 37].

MCPyV, like HPV, is a small, non-enveloped DNA virus with a circular, double-stranded DNA (dsDNA) genome surrounded by an icosahedral protein capsid [29, 30]. The 5.3 kbp viral genome contains the viral origin of replication, the bidirectional promoters for viral transcription [38, 39], as well as the early and late coding regions [29]. The early region encodes alternatively spliced tumor antigens, including Large Tumor antigen (LT), Small Tumor antigen (sT), 57-kDa tumor antigen (57kT), and the overprinting gene alternate LT ORF (ALTO) [29, 40]. The late region encodes the capsid proteins, VP1 and VP2 [41, 42].

LT and sT are the best-studied MCPyV proteins that have been shown to support viral DNA replication and MCPyV-associated tumorigenesis [30], whereas the functions and physiological significance of both 57kT and ALTO remain to be elucidated [29, 40, 43]. LT antigen stimulates host cell proliferation mostly through binding retinoblastoma protein (RB) using an LxCxE motif encoded within its N-terminal region [44]. It also drives the replication of the viral genome using the origin binding and helicase domain resided in the C-terminal region [39, 45]. MCPyVsT can robustly stimulate LT-mediated DNA replication [46, 47]. In addition, sT also promotes cellular proliferation by inducing hyper-phosphorylation of 4E-BP1 [48].

Similar to HPV, the genetic material of MCPyV is maintained as a circular episome during productive infection but is found to be integrated into the host genome in about 80% of recorded MCCs [28]. MCPyV-positive MCC tumors show a clonal integration pattern of the viral genome, suggesting that the integration event occurs early in oncogenesis, prior to the expansion of tumor cells [44, 49, 50]. A common feature of the integrated MCPyV genomes is the presence of mutations in the LT coding sequence which introduce premature stop codons that delete the C-terminal Ori binding and helicase domains [44]. The N-terminal portion of LT expressed in these tumors is referred to as LTT (tumor derived LT) and it retains the RB-binding motif, allowing the LTT mutants to disrupt the host cell cycle. Additionally, it has been demonstrated that these tumor-specific mutations do not disrupt the expression of sT. Continued expression of MCPyVsT and LTT is required for MCC tumor cells to survive [51, 52]. These findings provide strong evidence that MCPyV is a major causative agent of MCC [49, 50].

Nevertheless, it is important to note that Merkel Cell Carcinoma originating from MCPyV infection occurs relatively rarely. A possible explanation for the low incidence of MCC despite the prevalence of MCPyV infection is that two rare, independent events are required for tumor initiation and survival: MCPyV integration into the genome *and* a mutation involving MCPyV Large T antigen truncation that eliminates the virus' ability to replicate its DNA following integration [28, 44] (Fig. 13.2).

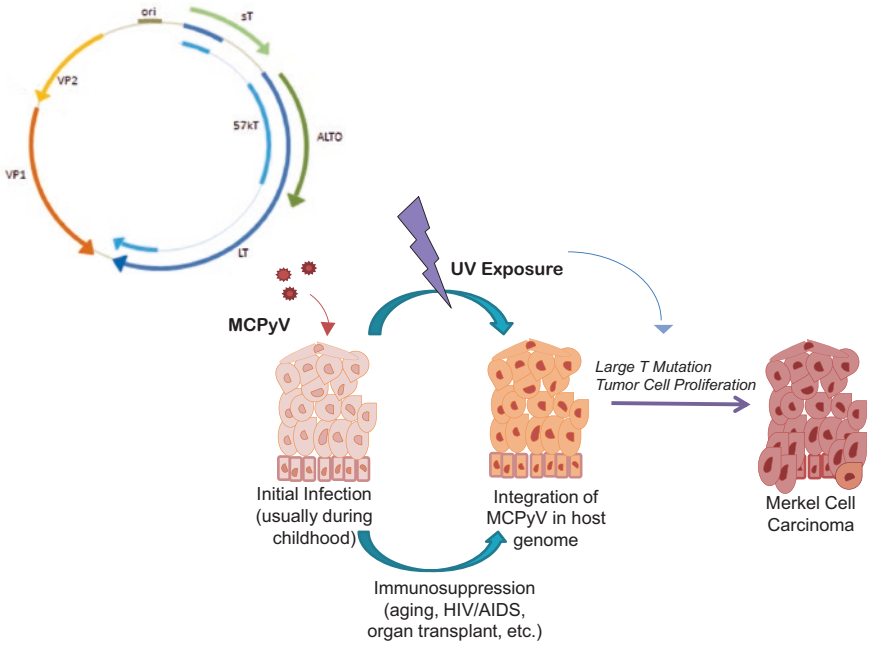


Fig. 13.2 The MCPyV genome and the progression of MCPyV infection resulting in oncogenesis. The MCPyV genome encodes early genes Large Tumor antigen (LT), Small Tumor antigen (sT), 57-kilodalton tumor antigen (57kT), and the overprinting gene, alternate LT ORF (ALTO). It also encodes the late capsid genes (VP1 and VP2). Of the early genes, LT and sT are the best-studied and are implicated in viral replication, carcinogenesis, and immune evasion. The non-coding region of the genome consists of a bidirectional promoter and enhancer elements along with the viral replication origin (*ori*). MCPyV typically establishes an infection during early childhood and is common in the general population. However, carcinoma resulting from MCPyV infection is rare. In order for this infection to progress to Merkel Cell Carcinoma, MCPyV integration into the genome and Large T antigen truncation are required. UV exposure and immunosuppression are key risk factors for MCC and may accelerate this process by promoting immune evasion and oncogenesis

13.3 Tumor Virus Infection and Associated Cancers in the Immunocompromised Population

13.3.1 Immunosuppression and Frequency of Viral Cancers

For both MCPyV and HPV, the virus-host balance that is achieved in a virus-infected cell is upended when the host immune system is compromised. In the case of HPV infection, pathogenesis is rare in healthy individuals, but several examples demonstrate that this is because an active immune system constantly works to suppress both cutaneous and mucosal HPV infections. For example, organ transplant recipients on immunosuppressive therapy frequently develop cutaneous warts and genital lesions [53]. In general, it seems that host immune responses do not prevent HPV infection, but restrict the ability of the virus to cause disease by limiting the viral DNA load in infected cells, by driving a reduction in viral protein expression via T cell surveillance, and more. Lesions arise more frequently in the immunocompromised population not because many more new infections take place, but rather because existing infections are less well controlled.

A similar situation exists for MCPyV-associated cancers. The incidence of many skin cancers is increasing among aging and immunocompromised populations, and although it is relatively uncommon, this is also true of MCC [54]. The increased incidence of MCC in immunocompromised patients indicates that both MCPyV infection and the host immune response are implicated in the pathogenesis and progression of MCC. Though the exact link between infection, a diminished immune response, and clinical outcome has yet to be established, clinical studies showed that immunosuppressed organ transplant recipients have a tenfold greater incidence of MCC than the general population. The incidence of MCC development is 30-fold greater than normal in chronic lymphocytic leukemia patients with a diminished cell-mediated immune response [55–59].

13.3.2 Tumor Virus Infection and Associated Cancers in HIV Patients

Immunosuppression as a result of HIV infection highlights the importance of immune control of both MCPyV and HPV. For the mucosal HPVs, several population studies indicate that HIV positivity is correlated with higher HPV prevalence. For example, rates of cervical HPV prevalence are about twice as high in HIV-positive as in HIV-negative women [60, 61]. This relationship between HIV infection and HPV infection is generally true for other populations and at other anatomical sites [62]. The factors that could contribute to these differences include a higher incidence of HPV infection, longer persistence of existing HPV infections, and

more frequent reactivation of existing HPV infections in HIV-positive individuals [62]. The higher incidence of HPV infection is observed in some, but not all, HIV-positive populations. Consistent with higher infection rates and with an effect of immunosuppression, HIV-positive patients with low CD4+ cell counts have higher rates of cervical lesions than those with higher CD4+ cell counts or than individuals not infected with HIV [63, 64]. There are also correlations between increased acquisition of HIV in HPV-positive individuals, but the evidence in support of this idea and the mechanisms involved are not as well understood as the HIV-mediated immunosuppression that leads to increased HPV infection [62].

Perhaps unexpectedly, there is a different distribution of high-risk HPV types in the lesions of HIV-positive women than in cervical lesions in the general population. HPV16 is the most frequent oncogenic genotype worldwide, but in HIV-infected women, there are proportionally fewer HPV16-positive lesions and more HPV18-positive lesions than in HIV-negative women [65–67]. The authors of these studies speculate that HPV16 is naturally better able to evade immune detection than the other high-risk viruses, meaning that the loss of immune surveillance resulting from HIV infection removes the greater restrictions on HPV18 and has less impact on HPV16 [62].

Similarly, the association between MCC and diminished immunity prompted researchers to study MCC occurrence among HIV patients. Patients with HIV have diminished CD4+ T cell counts and display cutaneous anergy resulting in an overall impaired immune response [68, 69]. One study by Engels et al. determined that HIV/AIDS patients have a 13-fold increased risk in developing MCC, adjusted for age and sex, with a significantly earlier onset relative to the general population [70]. It was found that the average age of MCC diagnosis among an immunocompetent population is 70, whereas for HIV patients, the average age of MCC diagnosis drops to 49 [71, 72].

Within the HIV-positive subset of patients, men with poorly controlled HIV infection had higher MCPyV viral loads compared to those with well-controlled infection [73]. The levels of MCPyV immunoglobulin G were also higher in HIV/AIDS patients than in either non-AIDS HIV patients or uninfected control patients [74]. While only 5.5% of the general population had $MCPyV^+$ blood serum, the viral DNA was found in the sera of 39.1% of untreated HIV-positive patients [75]. MCCs in AIDS patients are also characterized by aggressive clinical course with higher-grade lesions, more advanced tumor stage, and lower rates of survival [58]. These differences suggest that viral oncogenesis is more rapid and aggressive in patients with HIV-induced immunosuppression [76]. The elevated MCPyV DNA loads associated with HIV-induced immunosuppression may contribute to the increased likelihood of MCC development observed in HIV-infected individuals [76].

One of the classical risk factors for developing MCC is UV exposure as these lesions typically appear on sun-exposed areas such as the head and neck. However, in many HIV patients, these lesions frequently appeared on non-sun exposed skin, suggesting that UV exposure may not be the major risk factor in this population [34, 72]. Given both the atypical locations of tumor occurrence as well as the increased morbidity of this cancer among HIV patients, it is possible that MCC could possess

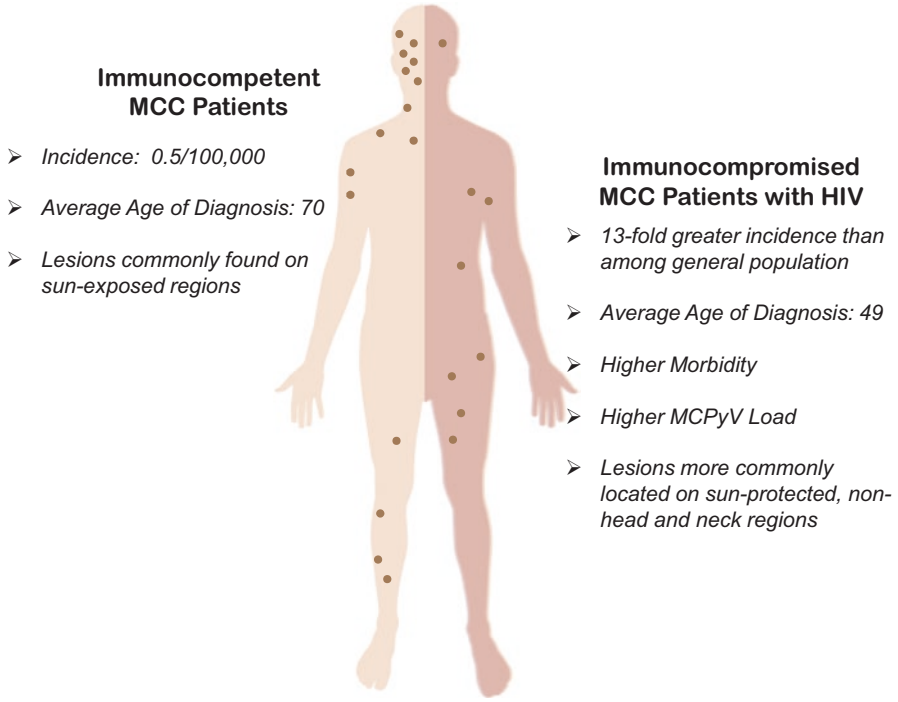


Fig. 13.3 MCC incidence in immunocompetent and immunocompromised patients. The increased incidence and morbidity of MCC among immunocompromised patients indicates that the interactions of MCPyV with a deficient immune system may result in a distinctive pattern of MCPyV+ MCC in the immunocompromised population

a unique pathology and pattern of progression among an immunocompromised population (Fig. 13.3).

13.3.3 Beta-HPV Infection and Epidermodysplasia Verruciformis

A link between HPV infection and cancer has also been proposed for certain beta HPVs and Epidermodysplasia verruciformis (EV) [77, 78]. EV is a rare genetic disease in which affected individuals develop frequent verrucous cutaneous lesions and are predisposed to develop squamous cell carcinomas that harbor high levels of these beta-HPV DNAs. EV is characterized by mutations in the *EVER1* and *EVER2* genes. It is clear that these mutations are related to immune function, although the precise mechanism is not yet understood [79–81]. In samples from a population that includes healthy individuals and organ transplant recipients there is a wide (seven orders of magnitude) range of beta HPV DNA load. Healthy subjects normally have

a viral DNA copy number of less than one genome per cell while immunosuppressed patients are more likely to have hundreds of viral genomes per cell [82].

13.4 Host Immune Detection and Evasion of the Host Immune Response

Each of the examples introduced so far illustrates that a healthy immune system is usually able to suppress disease caused by MCPyV or HPV infection. However, the ability of these viruses to persist emphasizes that they are well able to evade immune detection in order to achieve viral replication. We will highlight some of the similar and distinct mechanisms that are currently known to be used by the two viruses.

13.4.1 Human Papillomavirus Immune Evasion Mechanisms

The frequency of HPV infection and the long period of time over which infections persist both highlight the ability of the virus to evade immune detection. Several mechanisms by which HPVs evade immune detection have been characterized. It is apparent that these act during all stages of the viral life cycle, but it is not clear which mechanisms are conserved across diverse virus types versus specific for a subset of HPVs.

13.4.1.1 Early Defenses Against HPV Infection

HPVs are thought to access the basal layer of the epithelium via a microabrasion, or in the case of the cutaneous epithelium also via hair follicles. The structure of the stratified squamous epithelium is the first defense against HPV infection, as an intact epithelium does not allow access to the susceptible basal cells. Alpha-defensins and other antimicrobial peptides that are expressed by epithelial tissues have activity against several types of HPV pseudovirions (PsV) [83]. One of the peptides, HD5, was identified as one of the most active inhibitors among a panel of antimicrobial peptides and has subsequently been shown to inhibit HPV infection in two ways. First, it blocks the furin-mediated cleavage of the HPV L2 protein that is necessary for the early steps of HPV infection. In addition, it stabilizes and mislocalizes the capsid once the virion has entered the cell. This prevents the normal uncoating and trafficking of viral genomes [83–85]. HD5 has antimicrobial activity against HPV PsV of several virus types, but this mechanism of restriction is most relevant for the genital HPVs, since the HD5 mRNA is not produced in cutaneous epithelium even after stimulation of an immune response [86].

Other host defenses likely contribute to inhibition of initial HPV infection. A recent study [87] used PsV with capsid proteins from different HPV types to determine which interferon(s) restrict initial HPV infection. This study found that initial infection with HPV PsV is most inhibited by interferon gamma and not by any of several type I interferons or by interferon lambda. This is in contrast to the only other study that tested inhibition of PsV infection in the presence of interferon treatment [88]. In those experiments, interferons alpha and beta were the only two interferons tested and both were found to inhibit infection by PsV. Day and colleagues noted that the main difference between these two approaches is that the 2014 study used a luciferase reporter, meaning that both living and dead cells were assayed, while their 2017 publication used a GFP reporter so that only live cells were measured by flow cytometry. If interferon gamma-specific interferon-stimulated genes (ISG) indeed restrict early HPV PsV infection, the specific genes have yet to be identified and the mechanism of inhibition characterized.

It will also be important to determine whether components of HPV virions or the HPV early gene products are able to counter such inhibition. A study using canine papillomavirus suggested that papillomaviruses are able to dampen the interferon response triggered by incoming virus [89]. The authors of that study demonstrated that keratinocytes are competent to upregulate interferon beta, several chemokines and other ISG in response to stimulation with dsDNA or dsRNA. However, infection with canine papillomavirus type 2 (CPV2) did not upregulate the same genes, which they interpreted to mean that a CPV2 gene product inhibited the antiviral response. Overall, it is clear that host cells use several mechanisms to inhibit initial HPV infection and that there is more to learn about how these are countered by the virus.

13.4.1.2 HPV Interactions with the Intrinsic Immune System

There are several important intrinsic defenses that relate to HPV infection, and here we highlight two: ND10 bodies and a subset of the APOBEC proteins. Other contributors to intrinsic detection of HPV infection and the virus' countermeasures against them are discussed in more detail in [90].

ND10 bodies are punctate nuclear structures that are composed of several proteins with regulatory and immune responsive activities. The ND10 structure is assembled on a scaffold of PML protein [91] and the structure also contains reservoirs of transcriptional repressors Sp100 and hDaxx [90]. Although some ND10-associated proteins are induced by interferon, these structures and their components are also present in the un-stimulated cell and contribute to intrinsic immune responses. Many DNA viruses interact with ND10, either by depositing their genomes at ND10 sites or by altering the levels of ND10 proteins [90, 92]. For papillomaviruses, the genome together with the viral L2 protein are present at ND10 soon after initial infection [93, 94] although they may first be deposited near mitotic chromatin [95, 96] and then traffic to ND10 in a process that is mechanistically not understood. Subsequent transcription of HPV E1 and E2 genes leads to the formation

of HPV DNA replication foci at these sites. L2 is not strictly required for the formation of replication foci adjacent to ND10, since such foci are also observed in cells transiently transfected with HPV E1 and E2 expression vectors and a plasmid containing the HPV origin of DNA replication [94, 97–99].

Some components of ND10 bodies restrict HPV infection while others are required for infection to progress. PML and Daxx promote initial HPV infection and/or gene expression to varying degrees [93, 100–102]. In contrast, Sp100 restricts HPV infection at early and late stages of infection [102–104]. Altogether, the relationship between HPV and ND10 structures is different than for other DNA viruses. More experiments will be needed to determine how some ND10 components restrict HPV infection while others promote it and to understand the implications of these opposing effects.

With respect to cancer progression, PML was initially characterized on the basis of the chromosomal translocation that results in a PML-retinoic acid receptor alpha (RAR-alpha) fusion protein. This and other observations link ND10 components to cancer progression [105], but the function of ND10 bodies in HPV-positive cancer cells and in HPV-mediated carcinogenesis has yet to be determined.

Apolipoprotein B mRNA-editing catalytic polypeptide-like 3 (APOBEC3) proteins catalyze DNA cytidine deamination and conversion to uracil. They were first characterized as viral restriction factors and have more recently become appreciated as drivers of mutation in cancer. Similar to some ND10 components, APOBEC3 genes can be expressed at a basal level but upregulated upon stimulation by interferon, NF-κB signaling, and other stimuli. Individual cell types may express only a subset of the seven human APOBEC3 genes.

APOBEC3 enzymes act as restriction factors in HPV infection by altering viral genomes. APOBEC3-mediated editing of HPV genomes was first demonstrated by Vartanian and colleagues and has subsequently been shown to occur in other settings, including patient samples and additional HPV genome-containing cell lines [106–110]. A recent sequencing analysis examined over 5000 HPV genomes from cancer and non-cancer patient samples and found thousands of variant genomes [111]. Many of these genomes differ from the reference sequence with a signature suggesting that the mutations are the result of APOBEC3 activity. Perhaps paradoxically, both the high-risk E6 and E7 oncoproteins tested individually or in the context of the HPV genome cause an upregulation of A3A and A3B mRNAs and proteins [112, 113]. The A3B promoter regions that are responsive to E6 have been mapped and are responsive to TEAD transcription factors [114, 115].

Consistent with higher levels of APOBEC3 activity in the presence of high-risk HPV genomes, HPV-associated cancers also exhibit evidence of A3-mediated editing in the cellular genome [116–118]. However, some recent studies suggest that cancers with high mutation rates may provoke a heightened immune response and therefore have a better prognosis than those with fewer somatic mutations [119–121]. The relationship between APOBEC3 enzymes, HPV infection, and cancer is complex and is more extensively reviewed in [122].

13.4.1.3 HPV Interactions with the Innate Immune System

An innate immune response begins when a cell senses and responds to a non-self signal that is detected by a cellular pattern recognition receptor (PRR). Incoming pathogens are sensed by one of several arms of the innate immune system. Viral DNA or RNA is sensed by Toll-like receptors (TLRs), retinoic acid-inducible gene-I (RIG-I)-like receptors (RLRs), or the cGAS-STING pathway. TLRs are membrane-associated and sense foreign DNA at the plasma membrane or in endosomes, then trigger a signal that is transduced through MyD88 or TRIF and ultimately leads to the activation of NF-κB or IRF3/IRF7-dependent signaling pathways. The other pathways are active in the cytoplasm, where RLRs signal through MAVS and various DNA sensors signal through STING, again to activate IRF3/IRF7 and NF-κB. Activation of the IRF- and NF-κB-dependent signals results in the expression of interferons and some cytokines. These act via autocrine and paracrine mechanisms to induce a JAK-STAT signaling cascade leading to transactivation of ISG promoters by a STAT1/STAT2/IRF9 complex. Many of these pathways are recognized to be altered by tumor virus-encoded proteins [123] (Fig. 13.4).

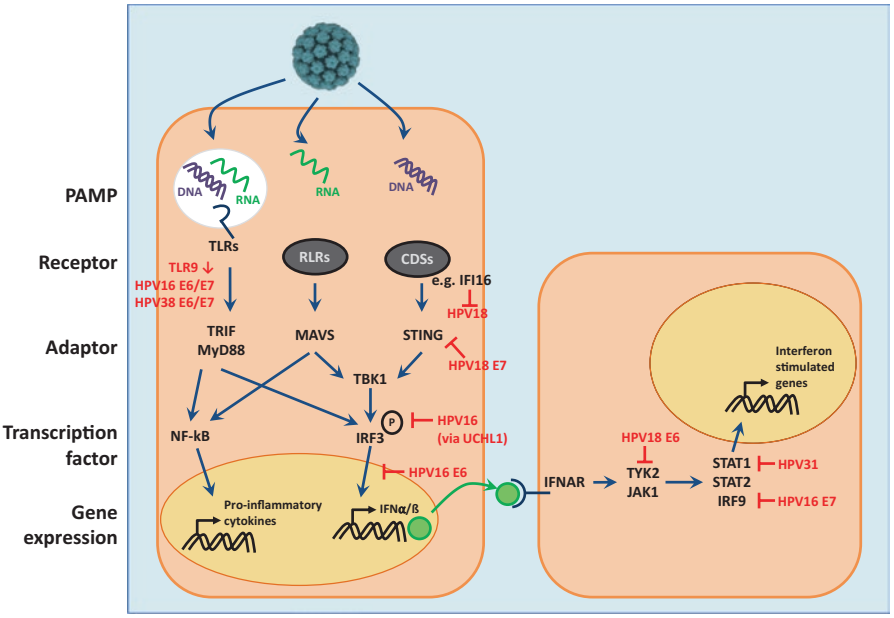


Fig. 13.4 HPV interactions with the innate immune system. Innate immune signaling is triggered by a pathogen-associated molecular pattern (PAMP), which is detected by a pattern recognition receptor (PRR) such as a Toll-like receptor (TRL), RIG-I-like receptor, or cytosolic DNA sensor (CDS). These transmit a signal via downstream adaptors to target transcription factors. Ultimately, foreign nucleic acid induces the expression of pro-inflammatory cytokines, interferons, and some interferon-stimulated genes. Interferons bind to receptors in an autocrine and paracrine way, stimulating JAK/STAT signaling and the transcription of additional interferon stimulated genes. Several HPV early proteins interfere with these signaling pathways

For the HPVs, global studies of gene expression in cells that produce one or more HPV proteins, harbor complete HPV genomes, or are transformed HPV-positive cancer cell lines emphasize that viral gene products can alter the expression of interferon, cytokines, and additional ISG [124–127]. One of the first such studies found that the presence of HPV31 DNA in keratinocytes resulted in a downregulation of several sets of genes and that one gene set was enriched for ISG [124]. HPV31-positive cells retained the ability to repress these genes even after treatment with one of several interferons. A similar analysis of HPV18 genome-containing cells compared cells with integrated HPV DNA to those that harbored HPV episomes. Both cell lines downregulated ISG and NF-κB targets and the integration status of the viral genome did not significantly affect this downregulation [126]. A study in which keratinocytes harboring HPV16 or HPV18 episomes were stimulated with polyI:C (a dsRNA mimic) generated a similar result [125]. A recent publication also examined the effects of keratinocyte differentiation on immune-related gene expression and again found that some genes are downregulated in differentiated HPV-positive cells [127]. Unlike the results from basal cells, that study reported that other genes including IRF1, interferon kappa, and genes encoding several viral restriction factors are upregulated in differentiated HPV-positive cells. Overall, there is a consensus that in basal cells one or more of the HPV early proteins acts to broadly downregulate the interferon response. Although these effects have been most frequently attributed to E6 and E7, other HPV early proteins likely have immune modulatory activity as well.

Interferon kappa is constitutively transcribed in keratinocytes and several studies have focused on this keratinocyte-specific molecule. A direct comparison of three different cell lines, each harboring a different high-risk HPV genome (HPV16, HPV18, or HPV31), showed that each of these viruses represses transcription of interferon kappa [128]. The authors of this study proposed that it is mainly the E6 protein that is mediating this response and that E6 is acting via transcriptional silencing. HPV31 transcription in episome-containing cells is inhibited by interferon kappa-mediated induction of Sp100 proteins [103]. Interferon kappa is silenced in cervical cancer cells [129]. Overall, it appears that dampening interferon kappa production and the resulting downstream signaling is important both for virus replication and for cancer progression.

As with many HPV studies, interpretation of these results is complicated by the use of different HPV types in different experimental systems, leaving questions about whether shared or distinct mechanisms are used by these viruses to alter innate immune signaling. Several of the individual mechanisms balancing HPV replication and innate immune restriction are discussed below.

DNA Sensors (TLRs and CDSs)

Several of the innate immune signaling pathways are active in keratinocytes, and HPV infection influences their activity. TLRs 1–6, 9, and 10 are expressed in keratinocytes [130], which can make differential responses to several different TLR

ligands. TLRs 1, 2, 4, 5, 6, and 10 are on present on the cell surface and TLRs 3 and 9 in endosomes. Each of these recognizes a different pattern foreign to the cell. TLR9 recognizes unmethylated CpG bases in foreign DNA and is the TLR that has been the most frequently studied with respect to HPV infection. One study reported that TLR9 is upregulated following keratinocyte differentiation [131]. Introducing HPV16 E6 and E7 into keratinocytes reduced the ability of keratinocytes to produce several cytokines in response to a TLR9 ligand and reduced the levels of TLR9 mRNA and protein [132]. HPV6 E6 and E7 had no effect and HPV18 E6 and E7 had an intermediate effect. A similar effect could be recapitulated with HPV16 quasivirus and required HPV16 E7 activation of NF-κB signaling [133]. Other reports do not agree that HPV infection causes a downregulation of TLR9. In a study of HPV-positive and HPV-negative cervical samples, TLR9 mRNA appeared to be uniquely and significantly upregulated in virus-positive samples compared to negative controls [134]. Other reports indicate that effects on TLR9 are not unique to the high-risk mucosal HPVs, as HPV38 also suppressed TLR9 expression at the transcriptional level by mediating the recruitment of a negative transcriptional regulatory complex to its promoter [135].

Other nucleic acid sensors are at work in keratinocytes. HPV18-episome-containing cell line models were used to show that IFI16 can restrict HPV18 infection via epigenetic modification [136]. Overexpression of IFI16 impaired HPV18 genome replication and viral gene transcription, whereas IFI16 depletion allowed HPV18 genome amplification to higher copy number. Marks of active transcription were decreased and repressive chromatin modifications were increased at the HPV18 early and late promoters in the presence of IFI16 overexpression.

Signaling Intermediates

HPV proteins also affect signaling through the pathways that are downstream of nucleic acid sensing and upstream of interferon production. Some HPV proteins target steps in the pathway that are common to both DNA and RNA sensing. An early study demonstrated that HPV16 E6, but not HPV18 E6, binds to IRF3 and blocks the production of interferon beta in response to Sendai virus infection [137]. Another study demonstrating effects on IRF3 found that the production of interferon beta and other cytokines is inhibited in the presence of HPV16 genomes when one of several different PRRs is triggered [131]. This group reported that upregulation of the ubiquitin carboxyl-terminal hydrolase L1 (UCHL1) acts through TRAF3 and TBK1, ultimately reducing phosphorylation on IRF3 and suppressing NF-κB signaling. Several mechanisms have been proposed by which HPV16 E7 might block transcription of ISG. HPV16 E7 was found to interact with IRF1 in a GST pulldown assay and to inhibit the transactivation of an IRF1-responsive interferon beta reporter plasmid [138]. IRF1 can activate a wide range of promoters including those for interferons and ISG. Similarly, an independent study found that HPV16 E7 inhibited the DNA binding activity of IRF1 and proposed that changes in downstream gene expression would result from this inhibition [139].

More recently, several groups examined the ability of HPV-encoded proteins to act on the cGAS/STING- or RLR-specific steps in the innate immune response. HPV18 E7 inhibits the production of interferon beta in response to transfected double-stranded DNA [140], a response that requires signaling through the cGAS/STING pathway. A recent study reported that HPV16 E6 and some other high-risk E6 proteins can also inhibit the RIG-I arm of the signaling response [141]. They do so by inhibiting RIG-I ubiquitination and its interaction with the downstream effector MAVS.

JAK-STAT

Several mechanisms have been proposed for HPV-mediated inhibition of components in the next step of the innate immune response, JAK-STAT signaling. HPV31 evades immune detection via transcriptional inhibition of STAT1 [124]. In contrast, HPV18 E6 has been demonstrated to bind TYK2, thus preventing the activation of the Jak-STAT pathway [142]. In a series of experiments examining the interaction of HPV16 E7 with IRF9, HPV16 E7 was found to inhibit interferon alpha signaling and *in vitro* binding experiments were used to demonstrate an interaction of HPV16 E7 with IRF9, proposing that this is the mechanistic basis for that inhibition [143–145].

Although there are many mechanisms by which HPVs inhibit innate immune signaling, HPV-infected cells remain sensitive to interferon treatment. The growth of HPV31 episome-containing cells cultured long-term in the presence of interferon beta is inhibited compared to the growth of HPV-negative keratinocytes [146]. These effects were minimized when HPV genomes were integrated, as in cancer cell lines, or when only the E6 and E7 genes were present. Consistent with this, interferon beta treatment of HPV16 episome-containing cells causes the loss of HPV episomes and the establishment of cells with integrated viral genomes [147]. Apparently, these cells arise from new integration events rather than selection for existing integrants [148]. Interferon treatment in clinical settings can be useful for lesions caused by low-risk HPVs, but has mixed results for the treatment of lesions and cancers caused by high-risk HPVs [149–154]. The authors of the studies in cultured cells speculate that interferon treatment in patients may actually drive HPV genome integration and clinical progression [147, 148].

HPVs use several mechanisms to evade detection by the innate immune system. Various activities have been ascribed to the viral early proteins from different virus types in distinct cell lines and experimental systems. Overall, the field lacks studies that directly compare HPV early proteins from different virus types in identical experimental systems. Performing experiments like these will help to determine the conserved or virus-specific nature of the mechanisms by which these HPV early genes impact immune signaling and to understand which targets might be relevant for therapeutic intervention.

13.4.1.4 HPV Interactions with the Adaptive Immune System

Epidermal keratinocytes contribute to the adaptive immune response. As previously introduced, they secrete interferon kappa [155], which recruits additional immune cells to sites of infection, and they also constitutively produce pro-IL-1beta, the precursor to the pro-inflammatory cytokine IL-1beta [156]. Epidermal keratinocytes express MHC molecules and have the capacity to present antigen [157], and MHC expression is reduced on virus-infected, including HPV-infected, cells [158]. For HPV16, this may be accomplished by the E5 protein [159] (Fig. 13.1).

Other cells in the epithelium are important as well. The majority of the 'professional' immune presenting activity in the epithelium is provided by Langerhans cells (LC), and there are fewer LCs in HPV-infected tissue than in the uninfected epithelium [159–161]. T cell activity in the epithelium is also important, and there are more T cells in the epithelium than circulating in the blood [162]. Some mechanisms by which HPV gene products might influence the composition of the T cell repertoire in infected tissue are emerging. A recent study of global gene expression data sets from cervical cancers of different stages and HPV-positive and -negative head and neck cancers noted a strong transcriptional downregulation of the CXCL14 gene [163]. This study primarily focused on cancer samples and found that epigenetic silencing of CXCL14 is responsible for immune evasion by HPV-positive cancer cells in a mouse model.

Because there are few tractable animal models for HPV pathogenesis, it is difficult to directly investigate how the adaptive immune response controls HPV infection. A recently identified murine papillomavirus (Mus musculus papillomavirus 1, MmuPV1) has been used to show the importance of T cell control of papillomavirus infection. With some mouse strain-specific differences, both CD4+ and CD8+ T cells contribute to the repression of productive MmuPV1 infection [164]. Altogether, it has been proposed that a T cell response does not eliminate HPV-infected cells from the epithelium, but rather drives them to limit viral gene expression in order to evade detection [165].

Finally, the immune response to the prophylactic HPV vaccines is primarily a B-cell mediated antibody response [166, 167]. Antibody titers following vaccination are several times higher than in a natural infection, likely owing to the route of immunization. Both B- and T-cell responses are able to protect against the consequences of HPV infection, but one is prompted by vaccination while the other is the result of a naturally occurring infection.

13.4.2 Merkel Cell Polyomavirus Immune Evasion Mechanisms

As previously discussed, MCPyV infection is highly prevalent in the general population [32, 168, 169]. These observations suggest that the virus has evolved a mechanism to escape eradication by the host immune system once it has established

latent infection. Furthermore, the incidence of MCC has tripled over the past 20 years due to an aging population as well as increases in prolonged sunlight exposure [54, 170]. Therefore, there is a growing interest in understanding how MCPyV interacts with host cell immune system in order to achieve and maintain persistent infection.

13.4.2.1 MCPyV Interactions with the Adaptive Immune System

Serological analyses revealed that as many as 88% of healthy adults are positive for MCPyV-specific antibodies [32, 171–173]. MCPyV DNA was detected in buffy coats of healthy blood donors and in inflammatory monocytes of MCC patients, suggesting that the virus may establish persistent latent infection in peripheral blood leukocytes [174, 175]. In two MCPyV-positive patients with inflammatory and non-melanoma skin cancer lesions respectively, MCPyV DNA was detected specifically in inflammatory, but not resident monocytes, indicating that the virus may persist in inflammatory monocytes and spread as these cells migrate through the body [174].

MCPyV-positive MCC patients display higher level of MCPyV-neutralizing antibodies than healthy controls but fail to inhibit MCC tumorigenesis [172], suggesting that humoral immunity is not sufficient to protect against MCC development. On the other hand, abrogated T-cell immunity has been associated with aggressive MCC outcome, arguing that the cellular immunity may play a more important role in MCPyV surveillance. MCPyV-specific T-cell responses are present in the blood of healthy individuals [176]. To specifically examine cell-mediated immunity against MCPyV, peripheral blood mononuclear cells (PBMC) of both MCPyV-seropositive and seronegative healthy adults were stimulated with MCPyV VP1 virus-like particles (VLPs) [177]. T-helper cell-mediated cytokine responses can be readily induced by recombinant MCPyV-like virus particle in both groups but much stronger Th-cell responses were detected in MCPyV-seropositive than MCPyV-seronegative individuals [177]. A robust IFN-γ response was also induced by MCPyV-specific Th-cells [177]. These findings support that Th-cells are the key mediators of MCPyV-specific immune surveillance.

13.4.2.2 MCPyV Interactions with the Innate Immune System

Several recent studies have begun to reveal the potential roles of MCPyV proteins in modulation of the host innate immune response. One piece of evidence supporting the role of MCPyV in evading the innate immune response is the downregulation of Toll-like Receptor 9 (TLR9) [178]. TLR9 is a critical sensor of the host innate immune response that recognizes both viral and bacterial dsDNA. It has been shown that expression of either LT or sT can inhibit TLR9 expression [178]. MCPyV LT contributes to this process by decreasing the mRNA levels of the C/EBPβ transactivator, which normally binds to a C/EBPβ response element (RE) in the TLR9 promoter to support its transcription [178]. This MCPyV function has been

suggested to allow infected cells to escape host immune surveillance [178]. However, whether MCPyV LT-mediated repression of C/EBPβ transactivator contributes to host innate immune evasion during MCPyV-associated tumorigenesis remains to be further investigated [178].

In a recent study looking at how the oncogenes of DNA tumor viruses target the host DNA sensing pathways [140], it was discovered that SV40 LT antigen expressed in immortalized mouse fibroblasts could inhibit the activation of both cGAS-STING and RIG-I pathways by their respective ligands [140]. This result suggests that SV40 large T may antagonize a common component of these antiviral responses. It will be interesting to test whether MCPyV LT has similar function and, if so, to determine the component(s) targeted by the LT antigens.

MCPyVsT also has been shown to down-regulate expression of cellular innate immunity genes downstream of NF-κB [179]. NF-κB family transcription factors can be activated by invading pathogens and inflammatory cytokines. NF-κB is normally maintained in an inhibitory cytoplasmic form in complex with members of the inhibitors of κB (IκB) family of proteins. Upon stimulation of the upstream signaling pathways, the IκB kinase (IKK) is activated, leading to phosphorylation and degradation of IκB. The released NF-κB can translocate to the nucleus and activate transcription of cellular genes involved in inflammation, immunity, cell death, and proliferation. sT was found to interact with NF-κB essential modulator (NEMO), thus inhibiting IκB phosphorylation and NF-κB nuclear translocation following stimulation with tumor necrosis factor alpha (TNF-α) [179]. Although this sT function has been suggested to constitute a mechanism by which MCPyV subverts the host immune response to allow establishment of persistent infection in the host cells [179], how sT modulates NF-κB signaling in the context of natural viral infection has not been determined.

In a separate study, microarray analysis was performed on hTERT-immortalized BJ human foreskin fibroblasts (BJ-hTERT) stably expressing tumor-derived LT, or co-expressing both tumor-derived LT and sT, to determine the differential host gene expression induced by these tumor antigens. This analysis revealed that expression of MCPyV LT and sT leads to upregulation of many cellular genes involved in cell cycle progression, DNA replication, and immune signaling pathways. Of particular interest, expression of MCPyVLT/sT antigens resulted in elevated expression of multiple IFN-induced genes, cytokines and chemokines [180]. In contrast, expression of tumor-derived LT with a mutated LXCXE motif defective for RB binding was not able to induce these gene expression changes [180], supporting an important role of LT-RB binding in MCPyV modulation of host gene transcription. It remains to be tested if upregulation of the immune signaling gene expression could also be observed in MCPyV-associated MCC tumors. If this is the case, the chemokines induced by tumor-derived LT/sT antigens might promote MCC invasion and metastasis [180].

Despite the progress made in understanding how MCPyV interacts with the host immune system, nearly all of the studies were performed using transfection or transduction of MCPyV genes into established cancer cell lines. How MCPyV interacts with the host immune system during natural infection remains unexplored. This is

largely due to the previously unknown MCPyV tropism and the technical difficulties in cultivating MCPyV in cell culture [42, 181, 182]. Recently, human dermal fibroblasts were identified as the likely natural host cells that support productive MCPyV infection [183]. The cell culture model for MCPyV infection established in this study provides a new opportunity to investigate the host immune response to MCPyV in the context of natural infection. It will be interesting to study how this virus-host interaction contributes to viral persistent infection and MCPyV-induced tumorigenesis.

13.4.2.3 Immune Escape Mechanism of MCPyV-Associated MCC

MCPyV integration is detected in nearly 80% of MCCs; however, in MCPyV-negative MCC cases, UV radiation is the primary source promoting oncogenesis [184–186]. While there are many differences between MCPyV-positive and -negative MCCs, both MCC subsets may be immunogenic and therefore can be targeted by immunotherapies now in development [186]. In MCPyV-positive MCCs, the viral antigens expressed are recognized by the host immune system as foreign and, by definition, make these tumors immunogenic. On the other hand, the high number of mutations observed in virus-negative tumors contributes to the higher neo-antigen burden observed in these tumors [184]. These features make both virus-positive and virus-negative MCC tumors ideal targets of the host immune system [184, 186].

The role of MCPyV in MCC development, along with the fact that immune-suppressed individuals are at higher risk for developing this cancer, indicate that host immune function plays a critical role in controlling MCPyV-induced oncogenesis [58]. Additional evidence such as the occurrence of complete spontaneous regression of primitive MCC and presence of tumor reactive T cells also highlight the function of the immune system in preventing and eliminating MCC [187, 188]. Furthermore, better prognoses are generally observed in MCC patients with robust immune responses, while high intratumoral MCPyV-specific CD8+ and CD4+ lymphocyte infiltration predicts better survival [189–192]. However, these T cells are only present in a very small percentage of MCC tumors [37, 190, 193]. In addition, immunosuppressed individuals only account for about 10% of MCC cases [57]; more than 90% of MCC patients have normal immune function but still fail to clear the MCC tumors that constantly express the highly antigenic, foreign MCPyV oncoproteins [37, 57]. MCC tumors continue to develop despite the presence of T cells recognizing MCPyV oncoproteins that are constantly expressed in the tumors [176]. These observations argue that immune evasion mechanisms help MCPyV-induced MCCs to escape immunological destruction.

One way by which MCPyV avoids detection by the immune system is through downregulation of major histocompatibility complex class I (MHC-I) [194]. MHC-I is necessary for presenting peptides from intracellular proteins to CD8+ T cells [195], making its downregulation an effective mechanism for immune escape. In one study, 84% of MCC tumors showed MHC-I downregulation, and MHC-I expression was lower in MCPyV-positive tumors than in those that were MCPyV-

negative [194]. These observations indicate that downregulation of MHC-I may be a mechanism by which MCPyV oncoproteins suppress recognition of virus-derived antigens by CD8+ T cells. In an analysis comparing gene expression profiles of MCPyV-negative and MCPyV-positive MCCs, Harms et al. showed that MCPyV-positive tumors maintained increased expression of immune response genes and enrichment of peritumoral CD8+ T lymphocytes [196]. This finding suggests a potential role of viral oncoproteins in modulating cellular immune response. However, much more remains to be studied regarding the immune evasion mechanism of MCPyV associated MCCs.

13.5 Therapeutic Implications of the Immune Response to Virus-Associated Cancers

13.5.1 The Impact of HPV Induced Immune Response on the Treatment of Head and Neck Squamous Cell Carcinomas

Between 60–70% of newly diagnosed cases of head and neck squamous cell carcinoma (HNSCC) in the United States are the result of HPV infection, whereas the remainder are not associated with HPV [197]. Consequently, it is possible to compare the large populations in each group and to consider the role of antiviral immunity in the response to cancer therapy.

The standard treatment for local HNSCC is either radiation or surgery [197]. The prognosis for HPV-positive HNSCC is generally better than that for non-HPV associated cancers, although HPV-negative versus HPV-positive HNSCC respond similarly to surgical treatment. In contrast, HPV-negative HNSCC respond significantly less well to radiation than do HPV-positive HNSCC [198, 199]. These observations so far are mainly from retrospective studies and meta-analyses, and the decision to treat with radiation vs. surgery is often based upon local expertise and preference at individual medical centers. There is a need for prospective trials to determine the best course of treatment for the two cancer types.

Several mechanistic explanations have been proposed to explain why the virus-associated HNSCC responds better to radiation. An appealing model is that the viral antigens that are released following the lysis of irradiated HPV-positive tumor cells provoke a heightened immune response compared to that generated by HPV-negative tumor cells [200]. However, there are many molecular differences between the HPV-positive and -negative HNSCCs that could also be important. For example, DNA damage repair is altered in HPV-infected cells and this may also make an important contribution to increased radiosensitivity. As for MCPyV discussed below, anti-PD-1/PD-L1 therapy has shown promise for the treatment of HPV-positive HNSCC and continues to be investigated in the clinic [201].

13.5.2 Therapeutic Approaches for Treatment of MCC

Currently, there is no effective treatment for metastatic Merkel Cell Carcinoma. Considering the immunogenic properties of MCC, many studies are looking towards immunomodulatory therapies as a potential solution. For both primary and metastatic MCC tumors, patients with higher level of T cell infiltrates and increased expression of immune response markers showed higher rates of regression and better prognoses [190, 202–204]. This correlation between prognosis and immune function supports the potential for immunotherapies in treating metastatic MCCs. As described above, MCCs, especially the MCPyV-positive cases, evade the immune response by down-regulating the expression of MHC-I [194]. Since this downregulation is reversible with interferon treatments, it has become a potential therapeutic target [194]. Stimulation of interferon production by the targeted delivery of the IL-12 gene using vaccination and/or electroporation is under investigation as a therapeutic approach [205].

Another promising approach for treating advanced MCCs targets the programmed cell death receptor 1/programmed cell death ligand 1 (PD-1/PD-L1) checkpoint. PD-L1 is overexpressed in some MCC tumors, especially the ones that are MCPyV-positive [186]. MCPyV-specific T-cells also express elevated levels of PD-1 [193]. Ligation of PD-L1 with the PD-1 receptor on the surface of T cells activates an immune checkpoint, which inhibits the anti-tumor immune response [206]. Therefore, anti-PD-1 therapy is an attractive treatment option for MCC [207]. In a recent study, patients treated with an anti-PD-1 antibody called pembrolizumab showed a response rate of 56% [208]. In 2017, avelumab, another immune checkpoint inhibitor targeting PD-L1, was approved as the first drug treatment for MCC. In a clinical trial involving patients with chemotherapy-refractive MCC tumors, avelumab had a response rate of 31.8% [209].

Although these immune checkpoint blockade strategies have shown promising results, the responses are short-lived, ranging in duration from 2 to 9 months [208]. Lesion recurrence is a concern and not all patients are responsive to this therapy [208, 210, 211]. These findings reveal the ability of MCPyV-associated MCCs to escape immunological destruction and resist immunotherapy, highlighting the need for understanding how MCPyV manipulates the host immune system in order to promote oncogenesis.

13.6 Open Questions and Future Directions

Despite the novel findings described above, many important questions remain in order to better understand the immune escape strategies employed by each of these viruses. For both MCPyV and HPV, the viral oncoproteins are not only specific to and important for the proliferation of MCC or HPV-positive cancer cells, but also naturally recognized as targets by the immune system, making them ideal targets for

immunotherapeutic treatment. It is not clear how viral evasion mechanisms contribute to the eventual tumor development induced by either virus. Because oncogenic viruses cause human cancer much more frequently in the setting of immunosuppression, it is of particular interest to determine if a lack of immune surveillance supports an unchecked viral replication, thereby inducing dysregulated host cell proliferation and cancer development. Since immunity to tumors overlaps with that of viruses, it is also possible that healthy immune systems can efficiently eliminate nascently transformed cells, but may fail to do so once compromised. If identified, the immuno-modulatory features of the viral proteins could be subverted to induce anti-tumor immune response against MCPyV-positive or HPV-positive tumor cells. Understanding how tumor viruses modulates the host immune system could help to develop more effective therapeutic strategies for highly morbid MCC and for the HPV-associated cancers that are a major medical problem worldwide.

It can seem contradictory to propose that the HPV and MCPyV early proteins that are best understood with respect to their role in transformation can be studied as immune modulatory proteins. However, there is emerging evidence that the p53 and pRb tumor suppressors that are well accepted as targets of the oncogenic viruses are themselves innate immune regulators [212–215]. One hypothesis goes further, proposing that the cellular targets of tumor viruses are in general proteins that are at the interface of cell cycle control and innate immune response pathways [27]. By targeting key cellular components that are shared by these signaling pathways, tumor viruses disable both the host antiviral and anticancer mechanisms, priming the infected cells for cancerous transformation. Many binding partners of HPV-encoded proteins have been identified in systematic analyses [216–218]. While some of these cellular targets are known to act in tumor suppressor pathways, others are not well characterized. It will be important to determine whether some of these affect immune responses and whether there is overlap between these immune-related activities and control of the host cell cycle. Since the limited coding capacity of the DNA tumor viruses drives them to target central nodes in cellular signaling pathways, the HPV and MCPyV targets may well be common targets of other pathogens as well.

Work to understand how HPV and MCPyV influence and are influenced by the microenvironment of the infected cell is still at a very early stage. Further development of new culture systems and animal models will help to explain the complex cellular interactions that impact the replication of these oncogenic viruses and their persistence in tissues.

13.7 Summary

Small DNA tumor viruses including MCPyV and HPV have genomes with limited coding capacity and are able to persist in infected cells over the long term. Persistence requires that the viruses efficiently evade immune detection, and some of the shared and distinct mechanisms by which they do so have been reviewed in this chapter.

We have highlighted some of the best understood ways in which HPV and MCPyV interact with and alter intrinsic, innate, and adaptive immune responses. We emphasize that the highly multifunctional proteins encoded by the DNA tumor viruses have historically been excellent tools to elucidate fundamental principles of cell signaling pathways. Studying them in the context of host immune responses will reveal new information as to the mechanistic basis of host immune responses and will inform therapeutic approaches to treat existing infections.

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Chapter 14
Innate Immune Pattern Recognition and the Development of Intestinal Cancer

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Abstract Inflammation and cancer have been connected since Virchow's pathologic examination of tumors revealed widespread immune cell infiltration. It is only recently, however, that a mechanistic understanding of this association has emerged. Pattern recognition receptors (PRRs), host receptors that transmit signals after binding moieties found in microbes or released by the host in response to injury, are one such molecular link. Recent work has established the importance of microbe-host signaling, mediated by PRRs, in a range of inflammatory responses, including the development and inhibition of cancer. Here, we review pattern recognition receptors and the implications of their activation on cancer. We focus on cancers in the gastrointestinal tract, the site of the greatest magnitude and diversity of the microbiota in humans. Signaling through PRRs impacts every stage of intestinal cancer, from the early phases of initiation to metastatic spread, and diverse cell types found in the tumor microenvironment, from neoplastic cells themselves to immune and stromal cells. We highlight recent discoveries that support a model in which tumors progress by exploiting PRR signaling. We argue that the tumor microenvironment exposes diverse signals from an altered microbiota and the host itself that converge on pattern recognition receptors, thereby perpetuating tumor growth. Analogous to pathogens, tumors orchestrate their own survival, which we propose occurs by both inducing and benefitting from alterations in host-associated microbial colonization.

Keywords Gut microbiome · Innate Immunity · Pattern recognition · Inflammation · Tumorigenesis

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14.1 Pattern Recognition Receptors

14.1.1 General Concepts and Specific Classes

Cancer disrupts tissue and organism-level homeostasis, providing a potent trigger for inflammation, a patterned set of host immune system responses that attempt to restore equilibrium [1, 2]. Infection and injury are prototypical sources of microbial- and host-derived signals that trigger inflammation. Pattern recognition receptors, evolutionarily conserved to recognize a diverse array of exogenous and endogenous ligands, drive inflammation by sensing these signals [3]. PRRs were initially theorized to respond to pathogen-associated molecular patterns (PAMPs), microbial structures that are not found in host organisms, thereby allowing for discrimination between host self and pathogen non-self [4, 5]. Importantly, these ligands are shared by non-pathogenic microbes. As such, PAMPs are generally referred to as microorganism-associated molecular patterns (MAMPs) [6, 7].

Pattern recognition receptors have been grouped into different classification schemes, and their definition has been expanded over time from the original proposed receptor that activated adaptive immunity through binding microbe-derived ligands [3]. Toll-like receptors (TLRs), human and murine homologues of the *Drosophila* Toll pathway, are the archetypal PRR family. Five additional broad families of PRRs have been described, including C-type lectin receptors (CLRs), nucleotide binding domain leucine-rich repeat (LRR)-containing (NOD-like) receptors (NLRs), RIG-I-like receptors (RLRs), AIM2-like receptors (ALRs) and cytoplasmic DNA sensors [8, 9].

14.1.2 Signaling Pathways

Not all host proteins that bind conserved microbial structures are pattern recognition receptors. Unlike antibodies or anti-microbial peptides, PRRs that are strictly defined activate downstream signaling cascades within host cells, rather than exerting their effects at the level of the microbe [8]. Signaling occurs in distinct cellular compartments, with receptor at the cell surface, bound on endosomes or cytoplasmic. TLRs and CLRs populate the membrane-bound compartments to monitor the extracellular environment, while NLRs, RLRs, ALRs and DNA sensors are cytoplasmic [8, 9]. Many PRRs across the different families rely on adaptor proteins to amplify their signals and promote downstream effects, often converging on the same enzymatic pathways as a result [10].

14.2 Inhibition of Tumorigenesis by PRRs

14.2.1 Immune Surveillance

The earliest descriptions of infections treating cancer predate modern germ theory. According to writings a millennium later, Egyptians from 2600 B.C.E. applied a poultice to an externally visible tumor and then cut the overlying skin, allowing cellulitis to develop and at times lead to tumor regression [11]. Coley's toxin, a mixture of heat-killed *Streptococcus pyogenes* and *Serratia marcescens*, provided in the late nineteenth century the earliest example of intentionally using microbial products to treat solid tumors [12]. Later work demonstrated LPS to be the bacterial component critical to the toxin's effects, as modest as they were [13]. Similarly, Bacillus Calmette-Guerin, the live-attenuated vaccine strain of *Mycobacterium bovis*, remains the standard of care in treating bladder cancer at certain stages of invasion, and has now been found to at least partly act by signaling through TLR2 and TLR4 to amplify antitumor cytokine responses and leukocyte recruitment [14].

Seemingly contradictory evidence has suggested roles for TLRs and other pattern recognition receptors in both initiation and arrest of gastrointestinal tumor development. The section below details the multiple ways PRR signaling initiates and promotes cancer development in the gut, while other evidence points towards protective effects of PRRs on tumors. For example, TLR signaling activates interferon pathways in dendritic cells to promote antitumor immunity in some cancer models [15], and PRRs can sense most oncogenic pathogens and can lead to effective host responses that clear these infections [2]. Modern studies have replicated Coley's work using purified TLR ligands that lead to tumor regression locally or systemically after inoculation [2]. Some have reconciled these data by splitting the PRRs into pro- and anti-carcinogenic, suggesting TLR2 and TLR4 act to promote tumors, including in the colon, liver and pancreas, while other TLRs, NLRs and inflammasomes are primarily tumor suppressive [15, 16]. Other explanations propose that the degree of stimulation influences how PRR signaling contributes to cancer. In this model, high levels of activation, such as by a high burden of replicating pathogens, stimulates acute inflammation that suppresses the active infection and can be harnessed for antitumor effects. In contrast, tonic low levels of PRR activation from the microbiota promotes chronic, low-grade inflammation that furthers tumor growth [15, 17].

14.2.2 Epithelium-Intrinsic Mechanisms

Disruptions in the colonic epithelial barrier are thought to be relatively early events in tumorigenesis [18]. The NLRP6 inflammasome may protect against colitis and inflammation-mediated tumorigenesis by maintaining this mucosal barrier [19]. NLRP6 facilitates mucus secretion into the gut lumen by promoting autophagy in

goblet cells, and is preferentially expressed in gut epithelial cells [20, 21]. Without NLRP6 signaling to generate functional IL-18, microbial composition is altered, with Bacteroidetes and TM7 phyla overrepresented, [20] in part through antimicrobial peptide secretion [22]. This altered microbial community, similar to that found in mice that lack the inflammasome adapter apoptosis-associated speck-like protein containing a CARD (ASC), can be transmitted to wildtype animals and subsequently predispose to colorectal tumor growth [23].

Other inflammasomes and cytoplasmic pattern recognition receptors also generally protect against cancers. Caspase-1 is a central effector and cysteine protease downstream of multiple inflammasomes that leads to cleavage and secretion of mature, proinflammatory IL-1β and IL-18, as well as mediating caspase-associated cell death (pyroptosis) and p53-related apoptosis. Multiple studies across animal facilities have demonstrated the importance of caspase-1 signaling in protecting against tumor formation in the setting of inflammation, but have separately proposed NLRP3 [24, 25] and NLRC4 [26] inflammasome signaling as the source of this protection, acting through IL-18 stimulating tumor suppressor production and epithelial-intrinsic injury response pathways, respectively. NLRP12, in contrast to other NLRs, limits inflammatory cytokine production, contributing to its protective role against tumor formation during inflammatory stimuli [27, 28].

Not all NLRs prevent tumors by altering the inflammatory tone of the intestines, but instead by maintaining normal epithelial-microbiota interactions. The NLR family apoptosis inhibitory proteins (NAIPs) form an inflammasome with NLRC4, but function independently of that signaling platform to protect against colonic tumorigenesis via p53-mediated apoptosis to remove damaged epithelia, limiting further dysplasia and degeneration into cancer [29]. The cytoplasmic peptidoglycan sensor Nod2, which detects muramyl dipeptide and ultimately leads to inflammasome activation, aids in maintaining a normal gut microbiota [30, 31]. In the absence of this NLR, the microbiota becomes altered and can be transmitted to other mice, displacing their indigenous microbiota. This altered community, in turn, promotes tumorigenesis in genetic models of colon cancer [30]. Furthermore, Nod2 limits TLR pathway activation, preventing inflammation-associated tumorigenesis [32]. NLRX1 is another NLR family member with similar protective effects in a non-inflammatory model of colorectal cancer by suppressing cellular proliferation that when unchecked leads to tumorigenesis [33]. Separate from NLRs, AIM2 detects cytoplasmic double stranded DNA, leading to caspase-1 activation during infection by recruiting ASC to form an inflammasome multiprotein signaling complex. Even without inflammasome signaling, however, AIM2 protects against intestinal cancer in colitis-associated models, instead limiting expansion of intestinal stem cells that fuel tumor growth [34, 35]. Mice without AIM2 develop an altered microbiota that contributes to tumorigenesis, as carcinoma formation decreases when the community is restored to that of a wildtype mouse [34]. Additionally, in a murine model of colitis-associated intestinal cancer, the double stranded RNA sensor RIG-I prevented changes in microbiota composition and tumorigenesis, [36] analogous to the protective effects of cytoplasmic inflammasomes.

14.3 Tumorigenesis Due to Pattern Recognition Receptor Activation

Colorectal cancer, a common source of mortality worldwide, follows a stereotyped pattern of development, classically described as the adenoma-carcinoma sequence [37]. From initial genetic stresses (initiation), activating mutations in oncogenes and, more commonly, inactivating mutations in tumor suppressors accumulate in a clonally expanded population of epithelial cells in a process known as tumor promotion. Initial hyperplastic, rapidly proliferating colonic tissue morphs into dysplastic adenomas that are often macroscopically visible in the colonic lumen as polyps. Once reaching a threshold of approximately 4–5 mutations, these tumors progress to a malignant phenotype [37, 38]. This patterned process provides the opportunity to study the contribution of different signals, including PRR activation, on each phase of tumorigenesis [39]. In humans, polymorphisms in TLR2, TLR3, TLR4, TLR5, MyD88, TIRAP, NLRP3 and IRAK2, among other signaling components, have been associated with colorectal cancer development, mortality or survival, indirect evidence of these pathways' importance [40–46]. At the protein level, TLR4 is overexpressed in inflammation-associated tumors in both humans and mice, in patients with ulcerative colitis and in mice treated with azoxymethane-dextran sulfate sodium (AOM-DSS) to induce tumors, respectively [47].

14.3.1 Tumor Initiation

Viral, bacterial and parasitic pathogens have long been known to initiate cancer formation, largely outside the gastrointestinal tract with the notable exception of *Helicobacter pylori* in the stomach [48]. Other microbes have been implicated in tumorigenesis more indirectly [49]. A subset of commensal *Bacteroides fragilis* expresses *Bacteroides fragilis* toxin (BFT)/fragilysin and is known as enterotoxigenic *Bf* (ETBF). This minor constituent of the microbiota causes colitis, colonic hyperplasia and subsequent tumor formation in mice by increasing Stat3 phosphorylation, thereby inducing T_H17 cells and IL-6 production [50, 51]. Abrogating these pathways decreased the number of tumors without changing their eventual size, arguing for a role in tumor initiation rather than progression [51]. How ETBF leads to Stat3 signaling remains unclear, but presumably is through a pattern recognition receptor [52]. IL-17 production from non-T_H17 cells, such as innate γδ T cells also contributes to tumor formation [53]. This indirect tumorigenesis pathway is in contrast to the more direct DNA damage from reactive oxygen intermediates produced by certain Enterobacteriaceae [54]. Whether these pathways are active in humans is not yet clear, but patients with colorectal cancer are more likely to carry ETBF in their stool [55, 56].

These studies on the role of ETBF in colorectal cancer initiation led to the alpha bug hypothesis, in which a microbe found in low abundance is instrumental in

promoting disease, both directly and by altering the composition of the surrounding community in the microbiota [57]. This concept is analogous to the keystone pathogen hypothesis first described in periodontitis, a chronic inflammatory disease of the structures supporting teeth mediated by biofilm formation. A single keystone organism, *Porphyromonas gingivalis*, cannot cause periodontitis alone, even when able to colonize effectively. Instead, *P. gingivalis* rearranges the biofilm community prior to the development of inflammation, even without reaching high density carriage itself [58]. This process leads to departures in the composition and function of the microbiota from non-diseased states, termed dysbiosis [59]. A third model suggests that certain bacteria such as ETBF serve as the "drivers" of initial tumorigenesis, promoting an altered microbiota with a distinct array of bacteria termed "passengers," able to exploit this altered environment to outgrow other commensals as well as the driver bacteria themselves [60]. Unlike the alpha bug model, under this hypothesis the passenger bacteria ultimately outcompete the bacteria that drive the initial tumor formation. This model has significant implications for studies of community composition in clinical samples, as the initiating microbes may be long outcompeted from the environment by the time patients come to attention and have stool samples or other specimens sequenced. The alpha bug and keystone pathogen models, similarly, suggest the causal agents in tumorigenesis and alterations to the microbiota are by their nature low abundance even at the time of causing DNA damage or other cellular stress that initiates tumors, leading to their potential to be overlooked in clinical samples.

Specific bacteria are involved in colorectal tumorigenesis even in the absence of clinically apparent colitis. *Fusobacterium* species are enriched in the microbiota overlying carcinomas relative to surrounding healthy colonic tissue, and found more frequently in stool samples from colorectal cancer patients than healthy controls [61, 62]. *Fusobacterium nucleatum*, typically resident in the oropharynx, is more prevalent not only in carcinomas but colonic adenomas, early stages of tumorigenesis [63]. In mice with activating mutations in APC that predispose to adenoma formation, *F. nucleatum* increased the number of adenomas in the intestines and promoted colonic tumorigenesis, without causing macroscopically visible inflammation, unlike ETBF [63]. The mechanism of tumorigenesis by Fusobacteria involves pattern recognition receptors and downstream inflammation, however, as these bacteria recruit myeloid cells to infiltrate adenomas and carcinomas, leading to pro-inflammatory NF-κB signaling [63, 64] dependent on TLR4 [65, 66].

Further evidence for the importance of PRRs in the handling of exogenous, genotoxic stress comes from the finding that mice lacking MyD88 are unable to maintain intestinal homeostasis both at steady-state and during radiation-induced injury, a potent carcinogenic stimulus. Mice deficient in this PRR signaling adaptor were more susceptible to epithelial damage and less able to repopulate intestinal crypts after radiation injury. MyD88 promoted the development of BrdU positive cells in the intestinal crypts [67]. Later work, however, revealed MyD88 does not formally lead to tumor initiation [68]. In a model of gastric cancer, hyperactivation of Stat3 promotes TLR2 expression in epithelial cells, signaling through which is required in gastric epithelial cells to stimulate tumor growth. Studies with antibodies

blocking TLR2 activation demonstrated roles for this pathway in both tumor initiation and progression [69].

14.3.2 Tumor Progression

While responsible for less than 5% of human colorectal cancers, hereditary cancer syndromes provide models to study tumorigenesis [70]. In $APC^{Min/+}$ mice, a truncation in one allele of the APC gene that is mutated in the human disease familial adenomatous polyposis leads to the spontaneous development of hundreds of intestinal polyps with progression to cancer. Using this model, studies of *myd88* deficient mice provided evidence for the importance of PRR signaling in both spontaneous and carcinogen-stimulated tumor expansion [68]. $APC^{Min/+}$ mice that were also deficient in *myd88* exhibited less anemia and mortality, as well as fewer and smaller polyps than their *myd88*-sufficient littermates. There was no difference, however, in the number of microadenomas between groups, suggesting that MyD88 was not required for tumor initiation, only progression. Signaling through the MyD88 adapter was also required for expression of NF-κB dependent tissue repair response genes such as *fgf10*, *cox2*, *mmp7* and *igf1*, suggesting a mechanism by which tumors depend on inflammatory signaling pathways [68]. Other work subsequently demonstrated that TLR4 and MyD88 were required for epithelial proliferation via EGFR signaling through amphiregulin, [71] and via MyD88 stabilizing the c-myc oncoprotein to promote ERK phosphorylation and downstream signaling [72]. MyD88 signaling, specifically through TLR2 and TLR4, is also required for progression of carcinogen-induced tumors in mice treated with oxazolone to induce colitis [73]. In these mice, considered a model of the human inflammatory bowel disease ulcerative colitis, these signals allow for IL-6 production from M2-polarized macrophages [73]. These effects correlate with the roles M2 macrophages have in wound healing, tissue repair and angiogenesis [74]. Furthermore, TLR2 and TLR4 activation in nascent tumor cells releases cytokines that facilitate metastatic spread [75].

14.3.3 Regionality of PRR Activation

The tumor microenvironment contains multiple cell types, including epithelial-derived tumor cells, differentiated epithelial cells such as mucus-secreting goblet cells and bone marrow-derived cells from the hematopoietic lineage [1, 76]. The bacteria that colonize the gut are frequently overlooked as additional residents of the tumor microenvironment, but can even be carried with metastases to distal organs [77]. PRRs are expressed not only on leukocytes but on other members of the tumor microenvironment, including epithelial cells [71]. Studies using bone marrow chimeras have established the dependence of PRR signaling on both hematopoietic and non-hematopoietic cells in different cancer models [17]. Microbes and microbial

products induce tumorigenic inflammation in all these colonic compartments. An altered microbiota allows for the outgrowth of *pks*$^{+}$ *E. coli* and *Enterococcus faecalis* that are directly genotoxic to the epithelium via colibactin and superoxide respectively, examples of alpha-bugs initiating tumorigenesis [1, 60, 78, 79]. These specific *E. coli* strains more commonly colonize patients with colorectal cancer than those with non-oncologic diseases [80, 81]. Also acting at the epithelium, enterotoxigenic *B. fragilis* (ETBF) permeabilizes the intestinal barrier through its toxin BFT, allowing for PRR ligands to penetrate into deeper layers to initiate and sustain tumor-associated inflammation [82, 83]. ETBF is also thought to activate pattern recognition receptors on colonic enterocytes, though only the activation of NF-κB has been shown directly [52, 84, 85]. Within the lamina propria, ETBF also drives changes in bacterial community composition and downstream inflammation through local induction of T_H17 cells [51]. Fusobacteria, furthermore, deactivate natural killer cells infiltrating tumors via binding of the adhesin Fap2 to the host receptor TIGIT, [86] but when monocolonizing gnotobiotic mice are insufficient to promote tumorigenesis [87]. There is no single signature of changes in the microbiota associated with tumorigenic inflammation, with different bacterial taxonomic groups, ranging from species to phyla, reported as being higher or lower in abundance in different studies [88, 89]. Different disruptions in the microbiota that converge on similar changes in the metabolism and function of the resident community likely contribute to tumor growth [6, 90].

Bacterial translocation across the colonic epithelium not only activates local inflammation but impacts distal tissues. On reaching the portal venous system of blood vessels that drains the intestines, bacteria and bacterial products first reach the liver. Carcinoma progression in the liver depends on the presence of bacterial PRR ligands. TLR4-mediated sensing of the microbiota promotes tumor progression in the diethylnitrosamine (DEN) carbon tetrachloride (CCl_4) model of hepatic fibrosis, inflammation and ultimately hepatocellular carcinoma [91]. The microbiota can have distal effects even within the gut, as biofilms and associated inflammation are found in nearly all tumors in the ascending (right) colon, often at sites distinct from the tumor itself [92].

The localization of PRR activation in tumorigenesis extends not only the transverse axis of the gut but also longitudinally. Density of bacterial colonization increases along the length of the intestines, correlating with rates of polyp and tumor development that are far higher in the human colon than the small intestine [60]. The concentration of PRR ligands correlates with bacterial density, suggesting that inflammation-dependent cancer growth may require the microbiota for persistent stimulation. The colon, lung and skin carry unique microbiota of varying density, and all have high rates of cancer that develops in the setting of chronic inflammation. By contrast, joints are similarly subjected to repeated trauma and inflammation, both physiologic and pathologic, such as in overuse and autoimmune arthritis. Cancer development in joints is exceedingly rare, however, raising the possibility that chronic inflammation in the absence of a commensal microbiota to provide PRR stimulation does not promote tumors [1]. One counterexample could be the liver, which readily develops tumors during chronic inflammation such as viral

hepatitis, and does not have a commensal microbiota, though the liver is constantly exposed to microbial products that transit the gut epithelium into the portal veins [93]. Hepatic PRR activation, therefore, provides evidence that tumors develop when inflammation and microbial products are present in the same location, not necessarily requiring active colonization or infection by live bacteria. Cancer, ultimately, is not only a disease of unchecked wound healing in response to inflammation [76], but requires additional pathways and stimuli, such as those provided by the microbiota, to fully co-opt tissue repair mechanisms.

14.3.4 Source of PRR Activation

In both inflammatory bowel disease and colorectal cancer patients, *E. coli* is found more frequently adhered to the mucosal surface. Secreted mucus keeps the epithelium relatively sterile in the healthy colon [94]. Alterations during chronic inflammation such as Crohn's disease, ulcerative colitis or tumor development may lead to enhanced binding to TLRs [95]. Intact, replicating bacteria need not necessarily transit the mucosal barrier to promote inflammation, however, as demonstrated by distal effects of LPS on the liver [93].

Bacteria often can be isolated from tumor tissue, their growth unchecked in the relatively immunosuppressed tumor microenvironment. Proliferating tumors quickly outstrip their blood supply despite angiogenesis, leading to hypoxia and necrosis within cancerous tissues, a rich growth medium for bacteria, particularly anaerobes [96]. Tumors, therefore, can create a niche for colonizing bacteria that increases the burden of PRR ligands that further stimulate tumor progression. Enrichment of specific microbes to the tumor microenvironment can occur via expression of specific glycans, such as Gal-GalNAc that is overexpressed in colorectal cancer tissue and bound specifically by the Fap2 lectin in *Fusobacterium nucleatum* [97].

14.4 Therapeutic Implications

14.4.1 Targeting PRR Signaling Pathways

With such broad effects on tumorigenesis that do not depend on a cancer's exact genotype, PRRs are an attractive therapeutic target. Antibody-based therapies that abrogate inflammatory signaling can have significant immunosuppressive effects, however. While less profound than the immunosuppression from cytotoxic chemotherapy, blocking activation of the innate immune system that is partly cell-autonomous and not dependent on leukocyte infiltration could eliminate the few immune protections these patients have left [98]. Inhibiting downstream pathways

including NF-κB, such as via IKK-β or other intermediates, would have similar drawbacks. Short-term inhibition could have a role as an adjunct chemotherapy, but long-term use as prophylaxis or suppressive therapy would sacrifice innate immunity against a range of infections, as well as surveillance of other tumors [99]. Targeting the microbes that stimulate pattern recognition receptors is another potential therapeutic strategy, as tested in enterotoxigenic *Bacteroides fragilis*. During a critical window early in adenoma development, clearance of ETBF colonization with cefoxitin prevented progression to carcinoma [100]. Antibiotics, however, are relatively untargeted, much like cytotoxic chemotherapy; the narrow window for intervention before the adenoma-carcinoma sequence becomes independent of ETBF stimulation further limits the utility of this approach. Other bacteria implicated in colorectal cancer may be responsive to antibiotics even farther along in tumor progression, though, as demonstrated by metronidazole treatment to clear Fusobacterial colonization from carcinomas in mouse models, even late in disease [77].

14.4.2 Chemotherapy

Traditional cytotoxic chemotherapeutics have been thought to function by directly interrupting cell-intrinsic processes such as cell division and nucleotide synthesis. Recent studies have expanded our understanding of how antineoplastic medications target cancer cells, highlighting the importance of PRR pathways [101, 102]. Disrupting the microbiota through antibiotic treatment or germ-free mice impairs the effects of the TLR9 agonist CpG oligonucleotide combined with anti-IL10R immunotherapy in mouse xenograft tumor models, including colon carcinoma [103]. TLR4 was required for effective antitumor responses, and purified LPS could substitute for the microbiota in enhancing them. The Gram-negative species *Alistipes shahii* was sufficient to induce TNF expression during CpG and anti-IL10R treatment. While TLR2 was not required for this combined immunotherapy to decrease tumor burden, Gram-positive bacteria contributed to TNF signaling, suggesting a contribution from other pattern recognition receptors that target structures other than LPS [103]. Immune checkpoint inhibitors, such as antibodies like ipilimumab, directed against CTLA-4, a negative regulator of T-cell activation, are a newer, more targeted class of chemotherapeutic that also depends on the microbiota for activity. In melanoma, sarcoma and colon cancer models, germ free mice and those treated with a cocktail of antibiotics no longer had improvement in tumor burden after ipilimumab treatment, which could be rescued by recolonization with specific *Bacteroides* species. These effects were partially attributable to signaling through TLR2 and TLR4 [104].

The microbiota also contributes to the cytotoxic effects of the chemotherapeutic oxaliplatin, a platinum compound that forms DNA adducts. In these experiments, MyD88 but not TLR4 was required for the host to sense the microbiota and potentiate oxaliplatin cytotoxicity. Platinum-DNA adducts formed after oxaliplatin

treatment independent of MyD88 activity, but intact PRR signaling was needed to activate myeloid cells to produce reactive oxygen species, thereby improving tumor killing [103]. IL-1 and IL-18 did not contribute to oxaliplatin efficacy, suggesting that other PRR pathways, such as TLRs, are important in mediating its effects [103]. In other mouse and human data, however, T_H1 inflammation, such as that elicited by TNF, has been linked to tumor progression and metastasis, calling into question the significance of these effects [98]. In other xenograft models, cyclophosphamide, an alkylating chemotherapy agent, spurred an altered microbiota characterized by decreased abundance of Firmicutes, while concurrently increasing translocation of Gram-positive bacteria, especially *Lactobacillus* and *Enterococcus* species, across the gut barrier. Viable bacteria from these genera were isolated from the mesenteric lymph nodes and spleen, and promoted T_H17 cell development that was necessary to target tumors. Antitumor effects depended on MyD88 sensing the microbiota, and they were absent in germ-free or antibiotic-treated animals but could be restored by adoptive transfer of microbiota-elicited T_H17 cells [105]. Specific members of the microbiota can have opposing effects, with *Fusobacterium nucleatum* found to signal through TLR4 to upregulate autophagy pathways in tumor cells, inducing resistance to cytotoxic chemotherapeutics [106].

14.5 Conclusions and Unanswered Questions

Microbes are involved in cancer through multiple mechanisms, from direct genotoxic or transforming effects of infecting pathogens to serving as an alpha bug, driver or passenger microbe disrupting the normal microbiota [17, 49, 57, 60]. These models, however, largely focus on the bacterial side of the equation, minimizing the effects of the tumor. In many cases, it is unlikely that these bacteria evolved to promote tumorigenesis, as the disruptions they cause in the microbiota and host epithelium do not change how well they colonize or spread. As a complementary model, we propose that tumors have evolved to exploit microbes to serve as tonic sources of inflammation that feed their own growth. Many pathogens exploit inflammation, mediated through pattern recognition receptor activation, to promote their own growth [107–110]. In this model, cancer cells, abetted by PRR-expressing leukocytes, co-opt similar pathways. Changes in the microbiota, therefore, may both lead to unchecked host cell growth and be a product of it. A single bacterial species may sometimes have outsized effects relative to its abundance, such as in the alpha-bug or driver-passenger hypotheses, but our model does not depend on a rare inflammation-stimulating organism. Rather, tumors may exploit the common final pathway shared among different altered communities that lead to inflammation [88].

Signaling through pattern recognition receptors may serve as a positive feedback loop for tumor development. Nascent cancers lead to altered epithelia and inflammatory infiltrates that promote dysbiosis and access of microbial products to otherwise sterile compartments, each of which increases signaling downstream of PRRs to promote more inflammation. While accounting for the role of the tumor on

microbiota composition and not only the converse, this model raises several questions. Do changes in the microbiota initiate or even perpetuate tumorigenesis, or are they a mere bystander during, or even an after-effect of, tumor growth? Why do so many tumors depend on NF-κB activation as a pro-inflammatory signal needed for survival? Why do some tumors appear to depend on the microbiota rather than somatic mutations to acquire that signal? How are the protective effects of some PRRs bypassed to modify the microbiota, and could they be restored as a novel therapeutic strategy? Answers are starting to emerge to how tumors alter the microbiota, and how that process impacts host metabolism, immunity, cachexia and other systemic symptoms, as well as further tumorigenesis, but far more remains to be uncovered [1, 17, 101, 111]. Understanding how tumors exploit microbe-mediated, host-sensed inflammation is an important step in designing the next generation of targeted therapies.

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Chapter 15
Microbial Metabolites in Cancer Promotion or Prevention

Kimberly Cox-York, Evan Stoecker, Alison K. Hamm, and Tiffany L. Weir

Abstract The trillions of microorganisms inhabiting the gut have been increasingly recognized for their role in modulating the health of their human host. They have been implicated in complex diseases such as obesity, cardiovascular disease and cancer. The metabolites produced by the gut microbiota have the potential to promote or prevent tumorigenesis, and influence cancer treatment outcomes based on their concentration, target tissue and utilization by gut inhabitants. The mechanisms by which microbial metabolites affect tumor dynamics are not fully established; however, emerging technologies continue to improve our ability to investigate these connections. In the interim, increasing research supports concerted efforts to promote a balanced gut microbiota for the prevention and treatment of multiple cancer types. This chapter introduces several classes of microbial metabolites and their mechanism of action with respect to cancer promotion and prevention.

Keywords Cancer · Colorectal cancer · Estrogen · Gut microbiota · Inflammation · Microbial metabolites · Phytochemicals · Phytoestrogen · Short chain fatty acids

15.1 Introduction

The gut microbiota, which includes the trillions of bacteria, fungi, and viruses that reside in the gastrointestinal tract, has recently become an intense focus of research because of its association, and potential causal role, in the development of numerous diseases. These organisms interact with humans to sustain health through their role in various physiologic processes.

For example, they aid in digestion, help maintain intestinal homeostasis, and regulate host systems such as immune function and metabolism [1]. Many of these

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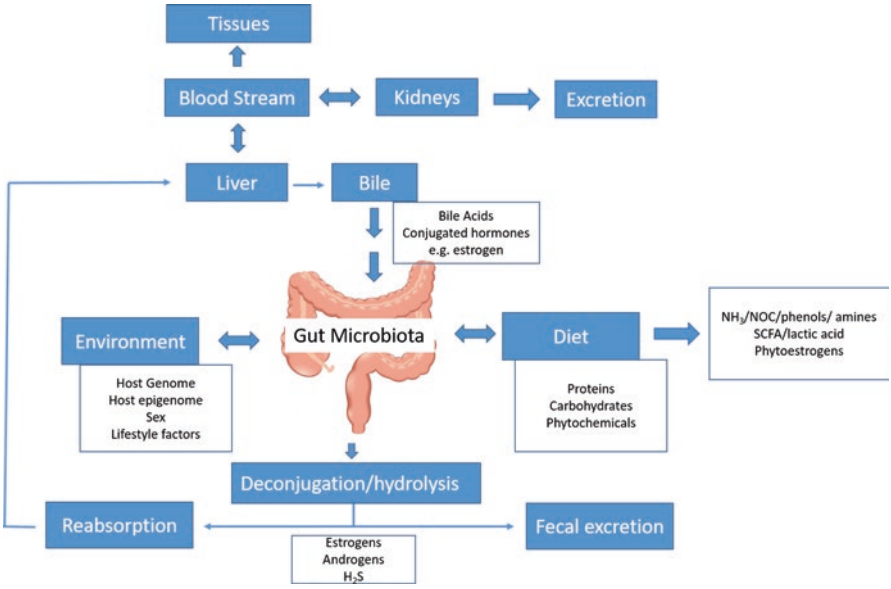

Fig. 15.1 Summary of interactions between environmental factors, diet and host-derived compounds and the gut microbiota

tasks are accomplished through the vast metabolic capabilities of these microorganisms. It has been estimated that the gut microbiota collectively may contain up to 100× more genes than the human genome [2]. Bacteria, which are the best studied organisms residing in the gut, contain genes that encode unique enzymes that would not otherwise be found in the host. These enzymes allow microbes residing in the human gut to break down food components that escape the human digestive tract, modify dietary components to alter their bioavailability and bioactivity, and metabolize host-derived compounds such as hormones and secreted bile salts (Fig. 15.1).

These microbial metabolites are important in the normal function of the gut and can act as signaling molecules to regulate host metabolic pathways. For example, propionate, a microbial end-product of fiber fermentation, can interact with G-protein coupled receptors on intestinal epithelial cells and other tissues, such as adipose tissue. This initiates pathways regulating lipid and glucose metabolism and stimulates production of hormones and other signals involved in satiety [3]. However, some microbial metabolites drive disease processes rather than assisting in the maintenance of human health. Microbial metabolism of dietary choline and carnitine results in trimethylamine (TMA) production in the gut, which is further metabolized by the liver into trimethylamine oxide (TMAO), a compound that has recently been implicated in development of atherosclerotic plaques [4, 5].

Microbial metabolism of some human-derived metabolites has also been implicated in cancer development. In the early 1970s it was postulated that steroid metabolism by the gut microbiota plays a role in cancer [6, 7]. Microbiota produce and metabolize hormones, and possess hormone receptors [8]. Moreover, there is evi-

dence that many of the enzymes involved in human hormone metabolism evolved from bacterial horizontal gene transfer [9]. At the same time, host hormones can affect the growth and virulence of bacteria [10]. The result of these interactions has direct and indirect implications for cancer development. In this chapter, we will highlight the microbial metabolism of dietary components and host-derived compounds (i.e. bile acids) that influence the development and progression of cancers, including colorectal, liver, and breast cancers (Table 15.1).

15.2 Fermentation Products

Microbiota accessible carbohydrates are dietary components that survive passage through the gastrointestinal tract and accumulate in the colon where they serve as microbial substrates. Simple sugars released from these complex carbohydrates are the preferred source of energy for most microorganisms in the gut, and the gut microbiota is particularly well-adapted to access these sugars. These microbes produce numerous enzymes capable of hydrolyzing glycosidic linkages to release simple sugars that can be metabolized by the bacteria for energy production. Fermentation of dietary fiber results in a variety of end products, depending on the metabolic capabilities of the specific bacteria present. The short chain fatty acids (SCFAs), butyrte, acetate, and propionate, as well as lactic acid, carbon dioxide, and ethanol are some of the major by-products of fermentation.

15.2.1 Butyrate

In bacterial cells, butyrate is primarily produced from an acetyl-CoA precursor via ATP generating mechanisms [11]. About 95% of all the butyrate produced stays in the colon where it is used as an energy source for healthy colonic epithelial cells [12]. Butyrate is a particularly important chemopreventive metabolite, and several studies have demonstrated an inverse relationship between fecal butyrate levels and colorectal cancer occurrence [13, 14]. Although butyrate serves as the primary energy source for colonic epithelial cells and stimulates colonocyte production under normal physiologic conditions [12], it is also an effective tumor suppressor due to its histone deacetylase (HDAC) inhibitory activity [15]. The ability of butyrate to both stimulate and suppress colonocyte differentiation under varying physiologic conditions is often referred to as the “butyrate paradox” [16, 17]. Under normal physiologic conditions, butyrate is taken into the cell from the luminal surface via monocarboxylate transporters and undergoes mitochondrial beta-oxidation and is used in energy production via the TCA cycle [12]. Cytosolic acetyl-CoA released during beta-oxidation can be used as a substrate for lipogenesis or as a cofactor for histone deacetylases. However, during tumorigenesis in colonocytes, cell metabolism changes from butyrate utilization to preferential use of glucose as an

Table 15.1 Microbial metabolites and their roles in various cancers

Metabolite	Associated cancer	Interaction/mechanism [references]
Short chain fatty acids		
Butyrate	Colorectal	Unmetabolized buyrate acts as HDAC inhibitor [15], Binds to GPR109A tumor suppressor [18]
Acetate and proprionate	Colon, hepatocellular carcinoma, glioblastoma, breast, prostate	HDAC inhibition, GPR43 tumor suppressor binding [20], tumor bioenergetics and proliferation via acetyl-CoA synthetases [23, 24]
Lactic acid	Colon	Tumor microenvironment is enriched in lactic acid bacteria, but no defined cause-effect has been established [26–28]
Protein catabolism		
Ammonia	Colorectal, breast	Increases pH of colonic microenvironment-colonocytes increase proliferation to adapt [37]; breast cancer cells assimilate ammonia for protein production [38]
N-nitroso compounds (NOCs)	Colorectal, esophageal, stomach	DNA alkylation [39–44]
Phenols and indoles	Various tissues	Activation of aryl hydrocarbon receptors-may cause damage or protection based on interaction and tissue [31, 45–50]
Amines	Colorectal	Genotoxicity due to nitrosation to nitrosamines [54]
Secondary bile acids	Gastrointestinal and hepatocellular	Activation of β-catenin pathway by conjugated bile acids can increase growth and invasiveness of cancer cells [66], damage ephtihelial layer leading to hyperproliferation of undifferentiated cellls [67], genration of reactive oxygen and nitrogen species resulting in DNA damage [68], hepatocellular mechanisms vary from nuclear receptor activation [71] to inflammation, oxidative damage, and transformation of compounds [72]
Sex hormones		
Estrogens	Breast, reproductive tract, colon, renal	Bacterial metabolism of parent estrogens (from gonadal and adipose tissue) may lead to increased estrogen reabsorption and metabolite ratios with implications for cellular proliferation, differentiation and apoptosis [137–142], likely due to selective estrogen receptor activity [155]
Androgens	Breast, reproductive tract, colon, renal	Gut microbiota deconjugate androgens and interconvert androgens and estrogens [134, 135]; the balance of the two likely plays a role in cancer development [132, 133], gonatodropins may also be rgulated via secondary signaling through microbial porduction of short-chain fatty acids [130, 131]
Phytoestrogens		

(continued)

Table 15.1 (continued)

Metabolite	Associated cancer	Interaction/mechanism [references]
Isoflavonoids	Breast, reproductive tract, colon	Generally believed to be protective via antagonism of endogenous steroid hormones [160, 163, 165], but may be time and tissue dependent [169]
Prenylflavonoids	Breast, colon, ovarian	Bacterial metabolites have antiproliferative effects via aromatase inhibition [177], xenobioteic detoxification, and inhibition of procarciongenic compounds [178, 179]
Ellagitannins	Prostate, breast, colon	Metabolites (urolithins) decreased proliferation, induce cell cycle arrest, and modulate MAPK and Wnt signalling [180, 187] as well as decrease inflammatory markers [186], NFkB signalling [162, 180], and angiogenesis [180]
Lignans	Colon, breast	Lignan metabolites act as antioxidants, selective estrogen receptor modulators, and aromatase inhibitors [160, 163, 189]

energy source, suppressing beta-oxidation and resulting in butyrate accumulation in the cells. Under these physiologic conditions, the unmetabolized butyrate acts as a HDAC inhibitor, preventing the silencing of tumor suppressor genes [15]. It is also the only SCFA known to bind to the tumor suppressing G protein-coupled receptor, GPR109A [18]. These activities of butyrate demonstrate the importance of butyrate in maintenance of colonic health and prevention of colorectal cancer.

15.2.2 Acetate and Propionate

In addition to butyrate, acetate and propionate are the major metabolic products of microbial carbohydrate fermentation in the gut. However, unlike butyrate their role in promoting or preventing tumorigenesis is much less clear. Both propionate and acetate enter circulation via the portal vein, as opposed to butyrate, which mainly stays in the colon. Propionate is metabolized by the liver, so only acetate can be found in significant amounts in systemic circulation [19]. Similar to butyrate, propionate has demonstrated HDAC inhibitory activity on colonocytes and certain immune cells [20]. In addition, propionate and, to a much lesser extent, acetate also bind to the G protein-coupled receptor, GPR43, which acts as a tumor suppressor [21]. Acetate has been shown to reduce colon cancer cell viability *in vitro* [22]; however, there is a large body of evidence that suggests that acetate serves as a fuel for tumor cells, promoting cancer progression. The nucleocytosolic enzyme, acetyl Co-A synthetase (ACSS2), can capture acetate to be used for the production of acetyl-CoA, which is a key metabolite in cellular bioenergetics and proliferation [23]. ACSS2 and other acetyl-CoA synthetases capable of capturing acetate have been implicated in the growth of hepatocellular carcinoma, glioblastoma, breast cancer and prostate cancer [24].

15.2.3 Lactic Acid

Numerous lactic acid-producing bacterial species (LAB) live in the human gut or are ingested with foods. In fact, most probiotic dietary supplements are comprised of lactic acid bacteria. These bacteria, and the lactic acid they produce, are generally thought to be beneficial and help maintain gut homeostasis and protect against pathogen invasions. These organisms have numerous types of special adaptations that help them withstand high acid environments, allowing the lactic acid to accumulate in their environment. Acidification by local production of lactic acid is a key factor in reducing pathogenic bacteria in fermented foods and protecting against gut pathogens. However, in a tumor environment lactic acid may stimulate the growth of cancer cells.

Tumor cells display altered metabolism whereby a high rate of aerobic glycolysis occurs. This metabolic shift, referred to as the Warburg Effect, redirects carbohydrates from energy generation into biosynthetic pathways, giving these cells a proliferative advantage [25]. The primary end-product of this glycolytic pathway is lactic acid. Although this metabolite was initially considered a by-product of cancer metabolism, new evidence suggests that lactate may directly contribute to tumor growth and progression [26]. While there is no evidence that lactic acid from LAB contributes to cancer development or progression, there have been reports suggesting that the colon tumor microenvironment is enriched in the lactic acid producing bacterium, *Streptococcus gallolyticus* (formerly *S. bovis*) [27]. This is likely the result of its high acid tolerance, rather than a cause and effect relationship, although this is still unclear. Tjasalma and colleagues have proposed the driver/passenger hypothesis for colorectal cancers, which suggests that an "alpha bug" induces tumor formation by producing genotoxic metabolites or generating reactive oxygen species, but is quickly displaced by "passenger" bacteria that are more metabolically adapted to the tumor micro-environment once tumorigenesis has occurred [28]. Thus, whether microbiota-derived lactic acid plays a role in cancer suppression or cancer progression is still uncertain.

15.3 Protein Catabolism

Although sugars are the primary fuel source of most bacteria, fermentation of peptides and amino acids is an important reaction, particularly in the distal colon where other preferred substrates may be limited. On average, the human colon encounters about 12 g of protein daily, of which ~50% is derived from the diet [29, 30]. Both host digestive enzymes and bacterial proteases and peptidases reduce this material to short peptides or component amino acids that can be used as substrates for bacterial fermentation. The end products of this fermentation include CO_2 and SCFAs, similar to carbohydrate fermentation. However, depending on the amino acids being fermented, other by-products including branched chain fatty acids, indoles,

phenols, H_2S, ammonia and amines are also produced through a series of chemical reactions such as deamination, decarboxylation, and α- and β-eliminations [31]. Therefore, while protein catabolism is a much less significant source of energy for colonic bacteria than carbohydrates, the putrefaction process- the degradation of proteins, can contribute to the production of systemic toxins that may influence the risk of developing colorectal and other cancers.

15.3.1 Ammonia

Ammonia is formed by the deamination of proteinaceous material. Its accumulation in the intestines is, in part, influenced by the rate of assimilation by bacteria for protein formation during carbohydrate fermentation, and its production through amino acid deamination reactions. Therefore, consumption of foods rich in indigestible fibers, which stimulate fermentation, have been shown to offset excreted ammonia resulting from high protein diets [32]. Several cell and animal models have suggested that ammonia can alter colonic epithelial cell function. Elevation of ammonia in rodent models has demonstrated reduced absorptive capacity [33] and decreased lifespan in colonocytes [34]. It has also been demonstrated that the highest ammonia concentrations and luminal pH in rats are associated with regions of colon where cell proliferation and aberrant cells were highest [34, 35]. Furthermore, elevated ammonia results in higher numbers of chemically induced tumors in rats [36]. Likewise, in cell models, ammonia favors the growth of tumor cells over normal healthy cells [35]. Only free ammonia is readily absorbed by cells, whereas in a healthy individual the colonic pH would be <7 and most ammonia would be in the non-absorbed form of NH_4^+. However, Fung and colleagues have proposed a model where low fiber, high protein diets create a "high risk" colonic environment that has greater exposure of the mucosa to ammonia and reduced levels of butyrate as a driver of colorectal tumorigenesis [37]. The role of microbiota-derived ammonia in other types of cancer is less clear, although it was recently demonstrated that breast cancer cells can assimilate ammonia for protein production, avoiding toxicity from the compound and turning it into a usable nitrogen source [38].

15.3.2 N-Nitroso Compounds (NOCs)

Nitrosation is the incorporation of NO to another organic molecule to form nitroso derivatives. N-nitroso compounds are genotoxic and carcinogenic and exposure can occur through ingestion in the diet or through endogenous production of these compounds in the stomach and intestines. The gut bacteria are an important aspect of this exposure as they can modulate levels of both nitrosating agents (NO derived from nitrate to nitrite conversion) and nitrosatable substrates (i.e. amines, indoles, and phenols resulting from protein degradation). Higher levels of these compounds

have been reported in diet interventions consisting of high protein and low carbohydrates, and NOCs were particularly associated with red meat intake [39]. The increase in NOCs with red meat rather than other animal proteins is likely due to the higher levels of heme proteins which are required for bacterial conversion of nitrates to nitrites [40]. NOCs have been demonstrated to cause cancer in numerous animal models [41]. One conclusion of the European Prospective Investigation into Cancer and Nutrition (EPIC)–Norfolk Study suggests that dietary NOCs are associated with higher incidence of gastrointestinal cancers, in particular rectal cancer [42]. Other prospective epidemiologic studies have also reported a link between NOCs and esophageal, stomach, colorectal, and rectal cancers [43, 44].

15.3.3 Phenols and Indoles

Degradation of the aromatic amino acids, phenylalanine, tyrosine, and tryptophan result in the production of phenols and indoles. Primary end products include p-cresol and phenylpropionate from tyrosine, phenylacetate from phenylalanine, and tryptophan is degraded to indole, indole acetate, and indole propionate by bacterial tryptophanases [45]. Like ammonia, excreted phenols have been associated with dietary intake of protein and are reduced by concomitant carbohydrate consumption, suggesting that some of these products are assimilated by gut bacteria during carbohydrate fermentation [31]. Stool concentrations of phenols and p-cresol, the detoxified form of phenols typically excreted in urine, are positively associated with colonocyte DNA damage [46]. In vitro, there is some evidence to suggest that phenols can be conjugated with nitrite to form genotoxic products and assist in nitrosation reactions of other metabolites [47]. However, there are few mechanistic links between phenolic compounds and cancer promotion, and numerous studies have suggested that phenolic compounds from plants are actually chemopreventive (see following Sect. 15.7 and [48]). On the other hand, there is a mounting body of evidence for a role of indoles in cancer pathology, primarily via activation of aryl hydrocarbon receptors (AHR) [49]. However, the specific role of indoles in cancer etiology appear to be determined by the indole compounds present, how they interact with the AHRs (agonists or antagonists) and in which tissue they are found [49, 50]. For example, indole activation of AHRs in the intestines appear to suppress inflammation [51] and colon carcinogenesis [52]; however, there are well established roles of AHR activation in mutagenesis and tumor formation elsewhere in the body [50].

15.3.4 Amines

Amines are produced in the decarboxylation of amino acids, and microbiota-derived amines found in the gut include agmatine, histamine, tyramine, and putrescine, among others. The physiologic role of amines in cancer development or progression

is largely unknown. However, putrescine has been demonstrated to regulate intestinal epithelial cell growth and differentiation [53] and derivatives of putrescine and cadaverine are reportedly excreted in higher levels by cancer patients than by healthy individuals [53]. Nitrosation of these compounds to nitrosamines may influence colorectal cancer risk as nitrosamine-containing fecal water extracts have displayed increased genotoxicity [54].

15.4 Bile Acid Modification

Bile acids are synthesized in hepatocytes using cholesterol as a precursor and are stored in the gall bladder. In humans, the primary bile acids produced are cholic acid and chenodeoxycholic acid, which are typically conjugated in the liver with taurine or glycine [53]. After consumption of a meal, bile salts are excreted into the duodenum where they are distributed throughout the small intestines to emulsify dietary fats and assist with their digestion. More recently bile acids have been identified as ligands for nuclear receptors such as farnesoid X receptor (FXR) and G-coupled protein receptors, acting as signaling molecules that regulate host lipid metabolism and may play a role in hepatobiliary diseases [55]. They are reabsorbed in the terminal ileum before being shuttled through the plasma and returned to the liver in a process referred to as enterohepatic circulation. Enterohepatic circulation of bile acids is heavily influenced by modifications made by reactions with intestinal bacteria. In the small intestines, bacteria deconjugate and oxidize hydroxyl groups on the primary bile acids to form secondary bile acids [56]. Only about 5% of bile acids escape reabsorption and enter the colon, but the secondary bile acids deoxycholic (DCA) and lithocholic acid (LCA) are mainly a result of bile modifications in the large intestine [57]. Lithocholic acid is poorly reabsorbed and is mainly excreted in stool [58]. Excessive levels of many bile acids have been reported in association with various cancers of the gastrointestinal tract and hepatocellular carcinomas, and these relationships were first identified as early as the 1930s. However, in this section, we will focus on secondary bile acids, DCA and LCA, and modifications resulting from microbial metabolism in the colon.

Bile acid conjugates, taurine and glycine, can be deconjugated and used as a substrate by multiple types of bacteria. However, hydrogen sulfide (H_2S) is released in the process of taurine deconjugation. H_2S has been shown to increase colonocyte turnover, potentially through up-regulation of ERK pathways [59, 60]. It can also prevent oxidation of butyrate, which is required as an energy source for colonocytes [61]. Furthermore, sulfide generation in the colon is associated with increased risk of chronic gastrointestinal diseases such as colorectal cancer [62] and individuals consuming a high risk “western” diet tend to have higher levels of taurine:glycine and hydrogen sulfide in stool [63, 64]. Thus, luminal levels of taurine-conjugated bile acids-which is mainly determined by diet, and the capacity of the gut microbiota to deconjugate taurine, may be important factors in determining colon cancer risk.

Case-control and epidemiologic studies have suggested a relationship between secondary bile acids, DCA and LCA, and colon carcinogenesis. Bayerdorffer and colleagues noted that unconjugated LCA was higher in serum of patients with adenoma [65]. Epidemiologic studies have shown that excreted LCA and DCA are higher with consumption of a high fat diet as well as in colorectal cancer patients [57, 58]. Mechanistically, there are several ways that bile acids, particularly secondary bile acids could influence cancer development. LCA has been shown to increase growth and invasiveness in cancer cells through activation of the beta-catenin signaling pathway [66]. Secondary BA's, particularly LCA, have also been shown to non-specifically damage the epithelial layer, and the subsequent repair mechanisms result in hyperproliferation of undifferentiated cells, which can create a precancerous state [67]. BAs also lead to the generation of reactive oxygen and nitrogen species that can lead to oxidative stress at the cellular level and directly induce DNA damage [68]. As a result, there is convincing evidence and plausible mechanisms by which secondary bile acids can influence development and progression of GI cancers. A review of the chemistry of various bile acids and how they may interact with the gastrointestinal tract to influence development of colorectal cancer was recently published [69].

Emerging evidence also suggests that bile acid dysregulation plays a role in other cancers as well. Hepatocellular carcinoma incidence is heavily weighted toward men relative to women, and animal models have demonstrated associations between the gut microbiome and liver cancers. The mechanisms appear to involve bacterial bile acid metabolism, which differs between males and females [70]. Mechanisms by which gut microbiota are involved in cancers of the liver include bile acid signaling mediated through interactions with nuclear receptors [71], inflammation, oxidative damage and transformation of compounds via deconjugation and dehydroxylation [72]. However, more research is needed in this area to conclusively establish a role for the gut microbiota.

15.5 Sex Hormone Metabolism

Sex steroid hormones (androgens and estrogens) are secreted into circulation primarily by the gonads, the adrenal gland and the placenta, and act both centrally and peripherally as transcription factors by binding to nuclear receptors [73], or as signal transduction activators via membrane-bound receptors [74, 75]. Hormones regulate a number of important physiological processes including development, reproduction, metabolism, homeostasis, inflammation, brain function, cell proliferation, differentiation and apoptosis [76]. They also facilitate "inter-kingdom" communication between microorganisms and their host [10]. This bidirectional interaction has been termed microbial endocrinology [77].

In addition to overtly sex-specific cancers (i.e. uterine vs. prostate), significant sex disparities exist in cancer incidence, tumor aggressiveness, prognosis and treatment responses in many other tissues [78–81]. Total and relative hormone profiles

are primary drivers in hormone-sensitive cancers [82–84], and are likely under the influence of gut microbial metabolism [85]; however, the degree of influence is debated. Some studies have shown little or no effect of sex on the gut microbiota [86–88], while others have found more positive associations [89–93]. In 89 inbred mouse strains, sex differences within strains were observed [85]. Gonadectomy and hormone treatment also resulted in microbial shifts associated with differences in bile acid profiles [85]. Additionally, a sex-by-diet interaction was detected when the animals were fed either chow or high fat diets. In humans, the dramatic change in hormone profiles of adolescence is associated with an increase in microbial diversity, defined by fewer aerobes and facultative anaerobes and increases in the number of anaerobic species [94, 95]. A recent study in middle-aged men and women also revealed a significantly lower abundance of Bacteroidetes in women relative to men [96].

Hormone-specific disease states, such as polycystic ovary syndrome (PCOS; a state of hyperandrogenism, menstrual abnormalities and polycystic ovaries) provide further evidence of a sex hormone-gut microbiota interaction. Gut dysbiosis correlated with disease state in human PCOS patients relative to controls [97]; and animal models of PCOS report diet-independent decreases in microbial diversity relative to control [98, 99]. Moreover, fecal microbial transplant from control donor mice and treatment with *Lactobacillus* restored the gut microbiota in a rat model of PCOS [99]. While not concrete, there is some evidence that women with PCOS have an increased risk for endometrial cancer [100, 101]. The androgen: estrogen profile is also evident in newly diagnosed liver cancer, which is 2.6 times higher in men than women [102]. A recent study of murine nonalcoholic steatohepatitis-hepatocellular carcinoma reported sex-specific differences in gut microbiota and bile acid retention, in accord with prior data implicating sex hormone receptor activity in hepatocellular carcinoma [103]. However, the complicated multistep processes involved in microbial metabolism of hormones and cancer development makes studying the link between the two a challenging endeavor.

15.5.1 Estrogens

The ‘unconventional estrogens hypothesis’ was proposed based on observations that circulating estrogens were composed of less than 1% of ‘conventional’ estrogens (estrone (E1), estradiol (E2) and estriol (E3) [104]. The liver oxidizes these parent estrogens (Phase-1 reactions) to form 2-OH, 4-OH and 16-OH estrogens, which vary from parent estrogens in bioavailability [105] and estrogenicity [106] at the canonical estrogen receptors ERα and ERβ. The ratio of metabolites to parent species is implicated in regulation of estrogen related diseases including cancer [107]. For example, the ratio of 2-OH, which has almost no estrogenic activity to 16-OH, having high affinity and agonist activity at ERα, has been associated with breast and endometrial cancer risk [108–112]. Phase-2 liver metabolism involves conjugation reactions, primarily glucuronidation and sulfation. The resulting

metabolites have little estrogen receptor binding capability and are subsequently incorporated into bile and urine, where they are subject to excretion. If not excreted in feces, estrogen conjugates may be deconjugated by microbial enzymes and reabsorbed, completing the enterohepatic circulation of estrogens. The deconjugation of estrogen species by bacterial β-glucuronidases and β-glucosidases [113, 114] may increase reabsorption and therefore circulation of free estrogens. Moreover, complex interconversions of estrogen metabolites have been confirmed by in vitro assays in human feces [115, 116]. Hence, a woman's lifetime estrogen exposure may be partially reflective of the attributes of her microbiome, and the modulation of this 'estrobolome' [117] may be critical in optimizing health-span.

As early as the late 1960s estrogen metabolism was inextricably linked to the gut microbiota. Stoa et al. [118] and Inoue et al. [119] described metabolite profiles specific to route of administration (i.e. oral versus intravenous) and Adlercrutz et al. [120, 121] observed differences in fecal and urinary estrogen metabolites after antibiotic treatment. Adlercrutz and colleagues then went on to demonstrate differences in estrogen metabolism between omnivorous and vegetarian women [122], suggesting that gut microbiota, and therefore estrogen metabolite profiles, are modifiable by diet.

Estrogens are known to regulate cell proliferation, differentiation and apoptosis-all important aspects of tumorigenesis. Estrogen may act locally or distally to influence development of neoplasms. Historically, estrogen has been linked to cancers of the reproductive tissues (breast, ovary, uterus and prostate), which are associated with concentrated levels of the canonical estrogen receptors, albeit in varying ratios of ERα: ERβ. Recent evidence supports a role for estrogen in non-reproductive tissues like lung, liver and the gastrointestinal tract as well [123–125]. Conversely, there is evidence that activation of estrogen receptor beta (ERβ) may play a role in the prevention of colon cancer [126]. The specific mechanisms by which estrogen modulates cancer risk have not been completely established and likely are nuanced based on factors such as sex, life stage, tissue type and other environmental exposures.

15.5.2 Androgens

Less is known about androgen metabolism by gut microbiota, however, like estrogens, androgens are inactivated via glucuronidation at the liver and are therefore subject to bacterial deconjugation in the gut [127]. Neonatal androgenization in rats results in decreased microbial diversity, and *Clostridium scindens*, found in the human gut, has the genetic machinery to convert glucocorticoids into androgens [128], highlighting the reciprocal regulation of gut microbes and steroid hormone balance. This interplay is further demonstrated in germ free mice, which display delayed testis development, lower circulating gonadotropins (LH and FSH), and lower intratesticular testosterone levels compared to specific pathogen-free mice [129]. These gonadotropins may be regulated via secondary signaling through microbial production of short-chain fatty acids [130, 131].

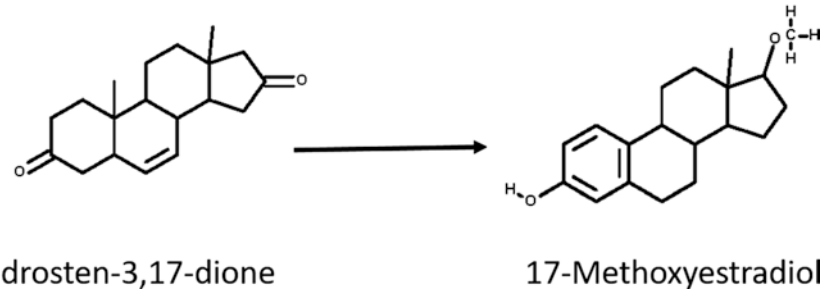

Fig. 15.2 Conversion of androgen to estrogen. The gut microbe *Clostridium paraputrificum* is capable of catalyzing this conversion in an NAD+ dependent reaction

The preponderance of evidence for the role of androgens, testosterone and dihydrotestosterone (DHT), in cancer is in the prostate, the primary target of androgen action. The mechanisms by which normal androgen signaling is disrupted, and prostate cells are transformed to become cancer initiating cells are unknown. Emerging evidence also points to a role for androgens in cancers of the female reproductive tract, including cancers of the ovary and endometrium, however, studies are few and the results are mixed. Given that androgens and estrogens often oppose one another in modulating physiological homeostasis, the balance between the two (and the respective receptors) may be more important than the prevailing profile of each individually [132, 133]. The balance of androgens to estrogens may be influenced by gut microbial composition. Gut microbiota produce several of the enzymes responsible for the interconversion of androgens to estrogens. This interconversion has been demonstrated in a strain of *C. paraputrificum*, which is capable of converting 4-androstene-3, 17-dione to 17-methoxy-estradiol in an NAD+-requiring reaction [134] (Fig. 15.2). There is some evidence associating *C. paraputrificum* and other *Clostridium* with colon cancer [135].

15.5.3 Tissue-Specific Associations of Sex Hormones and Cancer

15.5.3.1 Breast Cancer

Elevated circulating sex hormone levels are consistently associated with increased risk for breast cancer in postmenopausal women [136]. Total hormone levels have some predictive value, however, the relative concentrations of hormone metabolites may be the driving factor. In several studies, breast cancer risk is increased with increased levels of parent estrogens (E1, E2, E3), but reduced with increasing ratios of 2- and 4-pathway estrogen metabolites to parent estrogens, and with greater 2- versus 16-hydroxylation metabolites [137–139]. Recent work by Goedert et al. demonstrates a relationship between microbial diversity and estrogen metabolite profiles associated with breast cancer in postmenopausal women [140–142].

Since early observations of dietary influence on estrogen metabolism, specific pathways have emerged to describe the influence of diet on gut microbiome and cancer. Dietary fiber is of particular interest, and there appears to be a sex-specific response of the microbiome to total and specific fiber (e.g. from fruits and vegetables, grains, or beans) intake [96]. Dietary androgen has also been associated with estrogen receptor positive breast cancer risk [143]. The breast reportedly has a microbiome of its own, and dietary *Lactobacillus* species resulted in increased breast milk concentrations of the bacteria, and decreased staphylococcal count in 26- to 34-year-old women with staphylococcal mastitis [144]. These data further define the potential role of gut microbiota in breast health.

15.5.3.2 Reproductive Tract Cancers

Not surprisingly, sex hormone-associated cancer risk is highest in tissues of the reproductive tract, where sex hormone receptors are most concentrated. In postmenopausal women, endometrial/uterine cancer has been associated with increasing estrogen levels [145], and is nearly double in those in the highest quartiles of androstenedione, testosterone and DHEAS [145]. The same is true for ovarian cancer [146]. Direct links to the gut microbiome are difficult to make in these instances, because the reproductive tracts of both men and women harbor microbial communities of their own [147, 148], which have direct influence on gynecological cancers (reviewed in [149]). However, as has been discussed, the microbial metabolism of steroid hormones contributes to their systemic profile and potential for health consequences.

15.5.3.3 Colon Cancer

In 1971, Hill et al. published in the Lancet an association between fecal steroids and colon carcinogenesis [6]. Based on current statistics, colorectal cancer (CRC) occurrence is not different between men and women below age 40 years, but in adults 55–74 years, men have an almost 50% greater risk [150]. The disparity involves complex interactions between sex hormones and other risk factors. In populations from the Nurses' Health Study, the Women's Health Study, the Health Professional Follow-Up Study and the Physicians' Health Study II, estrogen: testosterone (E:T) increased relative risk of CRC in women, whereas higher total T and steroid hormone binding globulin (SHBG) and lower E:T ratio decreased risk in men [151]. Particular estrogen metabolite and SHBG ratios associate with different gut microbial communities [152], making it imperative to continue investigating the relationship between the two.

15.5.3.4 Renal Cancer

As with the other organs discussed, there is a bidirectional relationship between the kidney and the gut microbiota. Metabolites formed by the gut microbiota pass through the kidney in the process of blood filtration, while the filtration capacity of the kidney impacts the colonization of the gut via maintenance of intestinal tight junctions [153]. Renal cell carcinoma (RCC) rates in men are double that of women, and survival rates are best in younger women [154]. In vitro evidence suggests that 17β-estradiol may influence all stages of cancer progression in RCC, likely due to estrogen receptor expression profiles [155]. The relative expression of estrogen receptors differ between male and female rats [156], and changes along sex-specific lines with aging in mice [157].

15.6 Microbial Effect on Cancer Treatment Outcomes

In addition to its potential contribution to cancer initiation and progression, the constitution of the gut microbiota may also dictate cancer treatment outcomes. In an animal model of chemotherapy treatment, a pre-treatment microbial community high in *Lactobacilli* and *Enterococci* facilitated translocation of bacterial species across the gut epithelium, which was then detected in mesenteric lymph nodes and spleen within 48 h of treatment [158]. Overall, multiple lines of evidence point to a connection between gut microbial metabolism of sex steroids and several types of cancer. However, most evidence linking the microbiome and cancer is indirect, and there are many important questions to be answered [159]. Evolving technologies will allow these connections to be further defined and may lead to new prevention and treatment options.

15.7 Phytoestrogens in Tumor Prevention

Phytoestrogens are bioactive plant compounds, usually classified as polyphenols that exert effects similar to human hormones. These secondary metabolites are involved in protecting plants from ultraviolet radiation and pathogens, and assisting in mitigating effects of abiotic stress. Found in abundance in most parts of edible plants (especially those in the *Leguminosae* family) [160], these phytochemicals are common in the human diet. Phytoestrogens have been associated with numerous health effects in humans, including influencing cardiovascular, immune, and nervous system function as well as reproduction, skin, bone, and metabolism [160–162]. Phytoestrogens are structurally similar to 17-β-estradiol, as can be seen in Fig. 15.3, and impart both estrogenic and anti-estrogenic effects through binding to ERα and ERβ [161]. Once bound to an estrogen receptor, the phytoestrogen-receptor complex translocates to the nucleus and alters gene expression through

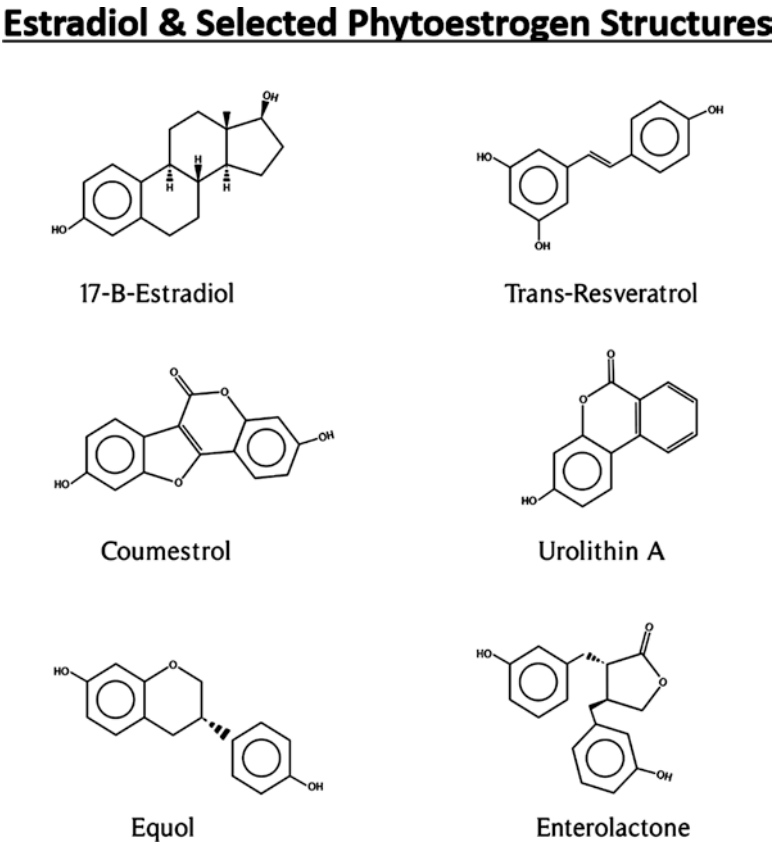

Fig. 15.3 Structural similarities exist between estrogen and several plant compounds. These phytoestrogens are capable of binding to estrogen receptors and display varying degrees of estrogenic activity

interactions with estrogen response elements or by binding early immediate genes. Phytoestrogens may also bind more specialized steroid membrane receptors that trigger rapid and transient non-genomic actions, such as increasing cAMP levels [161].

Phytoestrogens are categorized into five different classes: isoflavonoids, prenylnaringens, stilbenes, coumestans, ellagitannins, and lignans. Of these five classes, only isoflavonoids, prenylnaringens, ellagitannins, and lignans have known bioactive metabolites produced by the gut microbiome [162]. This is an important distinction as intestinal bacteria have been shown to have a strong influence on not only the bioavailability of phytoestrogens, but also their physiological activity and potency of action [162–164].

15.7.1 Isoflavonoids

Isoflavonoids are some of the most important, best-studied and controversial phytoestrogens with regards to their effects in human cancer. Twelve different soybean isoflavone isomers have been identified and include glycosides, acetyl glycosides, malonyl glycosides, and aglycones [162]. Aside from soybeans, isoflavones can be found in other legumes such as kudzu, lupine, and fava beans. Other foods may contain precursors to isoflavones, including chickpeas (contains biochanin A, a precursor to genistein) and alfalfa (contains formononetin, a precursor to daidzein) [161]. Associated processed products of the aforementioned foods have been shown to retain a majority of their isoflavone content [165]. Fermented products, such as tempeh or miso, are an exception, showing increased levels of isoflavones.

The major soy isoflavones, genistin, daidzin, and glycitin, are typically consumed in their glycoside form. The attached sugars make these compounds less bioavailable relative to their respective aglycones (genistein, daidzein, and glycitein), which lack the sugar moiety. As such, the bioavailability of glycosides relies upon their conversion to aglycones via beta-glycosidase in the tissue or from intestinal microbes [162]. Once the aglycones have been released, they are more readily absorbed through the gut epithelium and transitioned into peripheral circulation [162]. Not all isoflavones undergo transformation in the small intestine, however. Some isoflavones, together with an amount excreted into the small intestine from enterohepatic circulation, reach the colon unhydrolyzed. The mixture of isoflavone compounds, which may contain glycosylated, sulfated, and glucuronidated forms, are deconjugated by microbial enzymes in the colon. These metabolites of isoflavone compounds are either absorbed or further metabolized by the microbiota [162].

Isoflavones are generally thought to be protective against a few types of cancers including breast, colon, endometrial, ovarian, and prostate tissues [160, 163, 165]. Meta-analyses have noted a patterned reduction in prostate cancer diagnoses after the administration of soy isoflavones [163, 165]. Further, data from over 16,000 women was considered at the 102nd Annual Meeting of American Association for Cancer Research, where intake of soy was recommended as beneficial in regards to breast cancer [165]. However, there is some evidence that contrasts the proposed positive effects with regard to breast cancer and isoflavones, especially soy isoflavones [163, 166, 167]. Because isoflavonoids have been a significant and controversial point of focus in recent years, certain individual isoflavonoids, including genistein and equol, have been well-characterized with respect to their effects on human health. Genistein has received attention for demonstrating an ability to kill cancer cells, including breast and prostate cancer cells [165, 168]. However, genistein has been shown to be less effective in the presence of estradiol. Chen et al. [166] demonstrated the reduced efficacy of genistein in several protective capacities in the presence of estradiol in MCF-7 cells, and suggested that genistein and other phytoestrogenic compounds could actually stimulate cancer cell growth. The effects of genistein may be dictated by timing of exposure as a recent study suggested that lifetime exposure improved

response of chemically-induced mammary tumors in rats to tamoxifen treatment while later life soy exposure increased tumor growth [169].

Equol is a metabolite of daidzein and is exclusively produced by intestinal bacteria; however, only about 1/3 of individuals are equol producers [162, 165]. To produce equol, daidzein is hydrogenated to dihydrodaidzein which subsequently undergoes keto-elimination to equol. Dihydrodaidzein may also be converted to *O*-desmethylangolensin by ring cleavage. Equol's potential ability to fight cancer may be ascribed to its greater bioavailability relative to daidzein, as well as its anti-androgenic and antioxidant properties [162, 165]. Equol also has a chiral center in its heterocyclic ring, meaning it can exist as either a S- or R-enantiomer; however, the human microbiota can only naturally produce the S-equol enantiomer [162]. S-equol exhibits a preferred binding affinity for ERβ relative to daidzein. Once bound, its estrogenic activity is purported to be about 100 times greater than daidzein [165]. With respect to antioxidant activity, isoflavones, as a group, are often compared to vitamin E in terms of their antioxidant capacity [165]. Equol, though, has a greater antioxidant capacity than vitamin C or E [160]. Landete [162] proposes that the increased antioxidant potency of equol may be attributed to its increased flexibility compared to the more rigid structures of other isoflavones. The increased flexibility, a result of its nonplanar structure, allows equol to penetrate cell membranes more easily.

15.7.2 Prenylflavonoids

Prenylflavonoids are a group of phytoestrogenic compounds that include 8-prenylnaringenin (8-PN), 6-prenylnaringenin (6-PN), desmethylxanthohumol (DMX), and their precursors, isoxanthohumol (IX) and xanthohumol (XN). Prenylated flavonoids can be found in plants from the Cannabaceae, Guttiferae, Leguminosae, Moraceae, Rutaceae and Umbelliferae plant families; although those found in hops, *Humulus lupulus* (Cannabaceae) are the most studied [170]. 8-PN, XN and IX are only found in hops, and XN especially has been studied for its role in disease prevention, including cancer [171]. Both 8-PN and 6-PN bind to estrogen receptors, although 6-PN is a much weaker receptor agonist [172]. Neither compound is found in abundance in hops, but rather are primarily the result of spontaneous chemical reactions (i.e. isomerization) and microbial bioconversion. Unlike most phytoestrogens, 8-PN will preferentially bind to ERα compared to ERβ [173], and therefore may affect different tissues than other phytoestrogens.

8-PN is the most potent estrogenic compound among the prenylflavonoids but concentrations in animal models and humans is dependent on the gut microbiota. Individuals vary in their production of 8-PN, with differences in exposure attributable to variations in liver CYP450 activity and gut bacteria composition [174]. Ex vivo fecal incubations using samples collected from 51 individuals showed that ~20% of the participants lacked the ability to produce 8-PN while another 16%

produced very high levels [175]. In rodents and humans, the bacteria *Eubacterium limosum,* has demonstrated the ability to *O*-demethylate IX to form 8-PN [176].

Prenylnaringens can modulate hormonal signaling through interactions with estrogen receptors, but also through blocking aromatase, an enzyme responsible for synthesizing estradiol from androgens. XN, IX, and 8-PN have all exhibited ability to inhibit aromatase activity, which can potentially influence breast cancer development or progression [177]}. In fact, prenylflavonoids have been shown to inhibit growth of early-stage tumors, induce endogenous systems to detoxify xenobiotics, and inhibit activation of procarcinogenic compounds [178, 179].

15.7.3 Ellagitannins

Ellagitannins (ETs) belong to the hydrolysable tannin class of polyphenols and are derivatives of ellagic acid (EA) [180]. ETs are characterized by one or more hexahydroxydiphenoyl (HHDP) moieties esterified to a sugar, frequently glucose. The association of HHDP(s) with a sugar is the basis for the significant structural variety found amongst ETs due to the number of possible HHDP and sugar linkage sites [180]. These complex polyphenols are commonly found in fruits, nuts, and seeds such as pomegranates, raspberries, strawberries, walnuts, and almonds; they can also be found in a few beverages like cognac and oak-aged red wine.

While both ETs and EA have low bioavailability, they have been shown to be metabolized by gut microbiota in several mammals, including humans to form urolithins, which have increased bioavailability. Microbial metabolism of ETs and EA initially results in the production of urolithin C and D. These are subsequently conjugated in the liver to form urolithin A and urolithin B (the mono-hydroxylated analog of urolithin A) [180], which have increased lipophilicity and thus greater bioavailability relative to C and D [162, 180]. There is a large degree of inter-individual variability in the amount and type of urolithins produced, which is dependent on the host microbiota [181]. To date, only bacteria in the genus Gordonibacter have specifically been shown to produce urolithins [182, 183], although there are likely other sources due to the widespread number of producers found in human populations. Once formed, urolithins A and B remain in circulation from 12 to 56 h [180], during which time, they may interact with target tissues [184]. Gonzalez-Sarrias and colleagues confirmed urolithin A and B in the prostate of humans with urolithin A at a higher concentration than urolithin B [185].

Urolithins have demonstrated an ability to curtail the proliferation of cancerous cells, induce cell cycle arrest, and modulate key processes, such as MAPK signaling, in *in vitro* models of colon cancer [180]. Additionally, urolithin A has specifically been shown to decrease inflammatory markers such as cyclooxygenase-2, prostaglandin E synthase, prostaglandin E2, and inducible nitric oxide synthase in colonic mucosa in rats [186]. Lastly, urolithin A may inhibit the *Wnt* signaling pathway at concentrations that are physiologically attainable [187]. It has also been suggested that urolithins may help attenuate prostate and breast cancers. Regarding

the former, urolithins have been shown to inhibit nuclear factor kappa-B activation [162, 180], prolong prostate-specific antigen doubling times [188] and inhibit angiogenesis *in vitro* and *in vivo* [180]. With concern to breast cancer, Larrosa and colleagues demonstrated urolithins' ability to antagonize the growth promotion effect of estradiol in MCF-7 cells [186].

15.7.4 Lignans

Lignans are fiber-related diphenolic compounds that include secoisolariciresinol (Seco), matairesinol (Mat), pinoresinol (Pin), medioresinol (Med), lariciresinol (Lari), syringaresinol (Syr), sesamin (Ses), 7′-hydroxymatairesinol (7-Mat), and isolariciresinol (I-Lari) [162]. Plant lignans can be found in high to modest concentrations in many foods including, nuts/oilseeds, cereals/breads, legumes, fruits, vegetables, soy products, meat products, and alcoholic and non-alcoholic beverages, although flaxseeds are the richest source of plant lignans [162, 189]. They have poor bioavailability, but once metabolized to enterolignans by colonic microbiota, they are absorbed much more efficiently [162]. There are multiple and varied steps involved in the transformation of plant lignans to enterodiol and enterolactone, the primary lignans found in mammals. The transformational reactions may include deglucosylation, demethylenation, ring cleavage, demethylation, dehydroxylation, and oxidation [162]. While other factors such as diet and transit time are important to these reactions, the most critical is the composition and activity of the colonic microbiota [190]. The necessary microbes required for transformation of various plant lignan types into enterolignans have yet to be discovered. However, important identifications have been made, including those involved in the conversion of secoisolariciresinol diglucoside (SDG) to enterolactone [162].

SDG is the main lignan found in flaxseed and is first deglycosylated into Seco. Seco is then demethylated and dehydroxylated, progressively, to enterodiol. Enterodiol may then be dehydrogenated to enterolactone [162]. While these bacteria have been described as vital to the conversion of SDG to enterolactone, they may also be involved in the transformation of other plant lignans. For example *R. productus* also catalyzes the demethylation of Lari, Mat, and Pin [162].

Enterolignans are powerful antioxidants, selective estrogen receptor modulators with both agonistic and antagonistic estrogenic activities, and moderate to weak inhibitors of aromatase [160, 163, 189]. Plant lignin intake has been found to impact colon, breast, and prostate cancers. The antioxidant power of enterolignans prevents DNA damage and lipid peroxidation [162]. In a comparison between the antioxidant activity of Seco, enterodiol, enterolactone, SDG, and vitamin E, enterodiol was observed to be the most potent with an antioxidant potential more than five times higher than vitamin E. Seco, enterolactone, and SDG were 4.86, 4.35, and 1.27 times more potent, respectively, than vitamin E [191] .

Enterolignans are believed to be selective estrogen receptor modulators (SERMs). As such, they may fight cancer via anti-estrogenic actions once bound to estrogen

receptors. This may include competing with estradiol to bind estrogen receptors or initiating their own anticarcinogenic effects, e.g. recruiting transcriptional coregulators to associate with enterolignan-activated estrogen receptors [162]. It has been shown that physiologically attainable concentrations of enterodiol may activate estrogen receptor mediated events. With respect to more specific effects on certain cancers, the results of a few studies indicate that enterolignans may inhibit and/or reduce the incidence of colon cancer, especially enterolactone. Enterolignans, *in vitro*, can suppress the growth of human colon tumor cells [163]. Furthermore, while colon cancer progression is associated with the loss of ERβ, which is abundant in colon cells, enterolactone may help reduce ERβ losses [162]. Lastly, an assessment by Kuijsten et al. [192] of the association between plasma enterolactone level and incidence of colon and rectal cancer in over 57,000 patients between ages 50–64 concluded that higher enterolactone levels is associated with lower risk of colon cancer in women. Interestingly, these same enterolactone levels were associated with higher risk of rectal cancer in men.

In addition to colon cancer, there is research to suggest mammalian lignans may combat breast cancer. For example, adequate flaxseed intake is associated with a 20–30% reduction in breast cancer risk [189]. Additionally, it was observed that there was an associated risk reduction for anyone who had ever eaten flaxseed as compared to those who never ate it. While there are some conflicting studies about enterolignans' association with breast cancer risk, a meta-analyses of 21 studies found that high lignan intake was connected with a significant reduction in breast cancer risk in postmenopausal women [193]. Care must be taken, still, as the ability of mammalian lignans to induce estrogen-related genes may prove harmful in hormone-dependent breast cancer patients [162].

15.8 Conclusion

As has been discussed here, the microbiome assists in the metabolism of both dietary and host- derived compounds to exert effects on human health, including cancer (Fig. 15.4). Although a few relationships between specific bacteria and their metabolic by-products have been identified, there is still much work to be done in this area. These interactions must also be considered bi-directionally as the gut microbiota is a dynamic entity that is influenced by levels and types of substrates to which it is exposed. Often a lack of exposure to particular substrates will result in a reduced ability of the microbiota to metabolize that compound. For example, due to lifelong soy exposure, Asian populations tend to have a greater proportion of equol producers, presumably because the bacteria required for conversion have been selected for by diet. While cross-sectional and epidemiological data support a role for microbial metabolites in tumorigenic behavior, the evidence to date is primarily associative, and aside from diet, there are currently no approved therapies targeting the implicated metabolites. As tools to integrate global microbiome and metabolite profiling datasets improve, we will gain more insight into these important

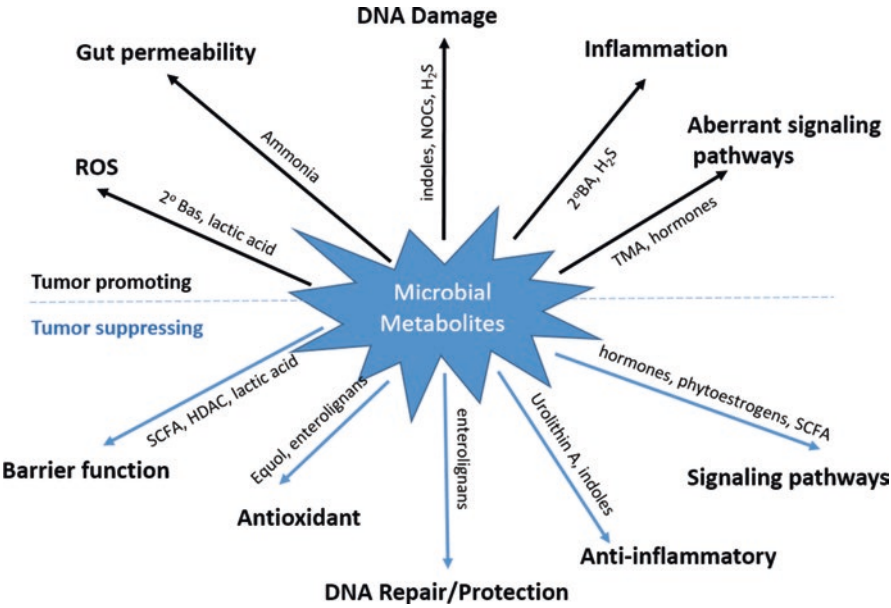

Fig. 15.4 Microbial metabolites of diet and host-derived compounds can suppress or activate various pathways involved in cancer initiation and development

microbiome-host-diet interactions. A better understanding of these interactions could help identify mechanisms of cancer development as well as provide new avenues of chemoprevention and treatment. While much research remains to be done, the prospects are nonetheless exciting.

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Chapter 16
Rapid Synthetic DNA Vaccine Development for Emerging Infectious Disease Outbreaks

Lumena Louis and David B. Weiner

Abstract Vaccines are considered among the top feats of modern medicine, saving millions of lives by inducing immunity to a number of infectious pathogens. As the next generation of vaccines seeks to address ever more complicated targets including cancer, innovative technologies like synthetic DNA vaccination that circumvent some of the issues associated with traditional vaccines will likely prove critical. In addition, compounding factors that may influence immune outcome such as the microbiome must also be studied in greater detail. Recent clinical studies have suggested that the presence of certain bacteria in the gut was associated with favorable outcomes in patients receiving immunogenic chemotherapy. Other studies have also shown that a dysbiosis or overrepresentation of other bacteria strains was negatively associated with favorable outcome. Further work needs to be done to more fully understand the influence that the microbiome exerts on the immune system and vice versa, and the significance of this relationship in designing future therapies.

Keywords Genetic adjuvants · Infectious disease · Electroporation · Cytokines · Microbiome · Intradermal vaccine delivery · Cancer · Immune-checkpoint inhibitors · DNA plasmid-mediated antibody (DMAb) · Gene therapy · Therapeutic vaccine

16.1 History of DNA Vaccines: New and Improved

Following the initial reports of DNA's ability to be used as an immunogen for generating an immune response over 25 years ago, significant work has been focused to realize DNA's intrinsic potential as a safe and potent vaccine platform in a variety of contexts. There has been a significant focus on both infectious diseases and cancer applications. While an enormous amount of exciting preclinical animal model

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data has been generated, until recently, while the DNA platform was safe, translation from small animal models to larger animals with robust immunity, as well as in the clinic was not achieved. However, recent advancements, including improved technologies for DNA delivery, improved concentrated formulations, improved stability of product, rapid production, improved construct sequence design including optimizations focusing on RNA changes as well as codon optimizations, and the inclusion of genetic adjuvants, have begun to establish this new synthetic DNA platform as a serious partner for rapid development for multiple applications and in particular for rapid protection against emerging infectious disease threats.

In the early 1990s, four separate groups reported that plasmid gene delivery resulted in *in vivo* expression and immune responses against the antigen. In 1992, Tang and Johnston reported the delivery of human growth hormone DNA to the skin of mice using a gene gun, believing that this could be a useful technique for gene therapy, however the gene therapy approach was not effective as the plasmid delivery resulted in antibodies against the HGH encoded protein. Separately, at the Cold Spring Harbor vaccine meeting in 1992, Margaret Liu along with her colleagues at Merck, as well as Harriet Robinson, from the University of Massachusetts, described DNA plasmid's ability to drive immune responses against influenza virus using plasmid delivered antigens, while David Weiner reported that plasmids encoding constructs for HIV or tumor antigens could induce neutralizing antibody responses as well as CTL's resulting in protection against tumor challenge. These three reports were soon published and stood as evidence to the vaccine field that a new technology consisting of deceptively simple DNA delivery could serve as a simple immunization platform in a number of models [1–4]. These early experiments in mice were to face immune potency issues over the next few years in larger animal models.

The vaccine field however, was excited by these initial studies. DNA vaccines had multiple conceptual advantages over traditional killed, live attenuated, and viral vector based vaccines. DNA is simple to work with, allowing for relatively easy manipulation for a variety of applications. DNA vaccines are nonlive and nonreplicating, eliminating the risk of attenuation/reversion and also allow for safe delivery in high-risk populations, including persons who may be immunocompromised. DNA vectors are themselves not immunogenic, allowing for repeated administration without immune interference or concerns regarding limited delivery due to previous viral exposure. In addition, DNA in theory can be manufactured to be more stable than traditional viral and killed vaccines thus possibly improving reliance on a complete cold chain, which in turn makes it an ideal candidate for important products developed for resource strained settings.

There are several important reviews that have elaborated on the mechanisms of the action of DNA vaccines, and so we will not discuss this aspect in fine detail here [5–12]. As an overview, DNA vaccines contain antigen sequences that encode for a particular part of a pathogen or tumor, designed to be inserted into a mammalian plasmid expression vector. The vector now becomes the new vaccine. Following production, this plasmid vector can be delivered intradermally or intramuscularly, i.e. locally to tissues, where upon cell entry, some of the delivered plasmid will enter the nucleus of transfected cells and plasmid-encoded sequences will drive host cell

transcription, producing the protein *in vivo*. This now *in vivo* produced foreign protein, can be expressed both in the transfected cells as well as released from these transfected cells to become recognized by B cells. The protein can become subject to immune surveillance allowing for presentation of this now foreign antigen on the Class I and Class II antigen presenting systems. This entirely native host system responds to this foreign antigen by eliciting a response including both antibodies (B cell responses) as well as driving cellular immunity (T cell responses), which can be protective in animal challenge models.

Due to the conceptual advantages of DNA vaccines over traditional live as well as nonlive platforms, and the success seen in most small animal preclinical models, excitement regarding the outcome of the DNA platform in humans seemed all but assured. However, as early human clinical trials failed to display the same level of immune response observed in preclinical studies, concerns mounted. The platform was well tolerated in people, but poorly immunogenic in the clinic. These results repositioned DNA to take a backseat as a primary immune approach, and opened up a new secondary role for DNA vaccines as a component in prime boost model systems. In these systems DNA is used as an initial priming immunization to focus and jumpstart the immune response, and then either protein or viral vector is used in subsequent boosting immunizations [13–22]. This combined approach led to greater immune responses compared to either platform alone and helped the viral vector approach to partially avoid the host immune response.

These early studies of DNA vaccines have since been reexamined and reengineered. The initial vaccines utilized dilute formulations of DNA, limiting the DNA dose that could be delivered, thereby limiting the efficacy of the vaccine. Today, due to new formulations [23–25], much more highly concentrated DNA is utilized, at doses upward of 10 mg/ml, which can increase vaccine efficiency. In addition to being more concentrated, newer formulations can be developed that are much more stable, reducing the need for complete cold chain transport, broadening the use of this approach in resource strained settings where total refrigeration or freezing may present challenges.

16.2 Electroporation Technology: An Electric Solution to an Old Delivery Problem

However, an additional major advancement is the use of new more potent delivery technologies combined with the new formulations. Specifically, the use of newer and reengineered electroporation (EP) devices to enhance *in vivo* transfection of delivered DNA during immunization, can result in a 100–1000× increase in transfection efficiency [26]. The application of an electric field immediately upon DNA injection enhances DNA uptake in two ways: EP creates transient pores in the membrane where the DNA can enter the cell and also generates an electric field to drive the DNA in to those cells as well. These activities combine to boost DNA uptake, creating a large bolus of now foreign protein *in vivo* ultimately driving

improved immune responses against the vaccine. Although older electroporation was initially considered too harsh to routinely use in humans, advances in EP technology that have generated computer driven devices, lowered voltages, and controlled current and timing settings, have all led to a more tolerable experience in people, making EP a viable candidate in vaccine development. As a consequence of these advances, delivery of DNA vaccines by EP in large animal models has led to increased cellular and humoral immune responses, rivaling those seen with viral vectors. There are a number of electroporation devices currently in use in both animal models as well as human clinical trials that all differ in their parameters as well as targeted tissue. Importantly, advanced EP that takes advantage of higher concentrated formulation and targets skin delivery may be particularly relevant for emerging infectious diseases (EID) settings. The simplicity and consistency of this combined DNA delivery approach in the clinic is a very exciting development.

16.3 Harnessing the Immune System's Messengers as Potential Adjuvants to DNA Vaccines

Adjuvants have had a long history in the vaccine field, primarily used to increase immunogenicity of various vaccines. Formulated adjuvants can function through a number of mechanisms, including enhanced antigen uptake and presentation, antigen depot formation, and activation of the innate immune system. Alum is currently the most widely used adjuvant in licensed vaccines, and while it has been successful at increasing vaccine responses, alum mostly enhances Th2 humoral responses, thus limiting its use in vaccine platforms where enhanced cellular responses are desired. Oil-in-water emulsions have also been studied as potential adjuvants. AS03, made by GSK, contains α-tocopherol and squalene, and has been shown to enhance vaccine specific humoral immune responses by increasing antigen uptake and presentation [27]. In the clinic, AS03 was incorporated in the pandemic H1N1/2009 vaccine and showed increased immunogenicity compared to non-adjuvanted vaccine. AS04, an adjuvant that is comprised of monophosphoryl lipid A and alum, is licensed and used in the human papillomavirus (HPV) vaccine Cervarix. A number of nontraditional adjuvants are being investigated as well, including pathogen-recognition receptor (PRR) agonists, nanoparticles, liposomes, and gene-encoded adjuvants [28]. PRR agonist adjuvants, including Toll-like receptor (TLR) ligands, exploit innate immune signaling, jumpstarting the body's first line of defense. This in turn can work in concert with the adaptive immune system to generate lasting memory against the antigen. TLRs are generally expressed by macrophages and dendritic cells that are constantly surveying for conserved pathogen associated molecular patterns (PAMPs) derived from microbes that breach initial physical barriers [29]. Their role for enhancement of gene encoded vaccine remains to be determined.

Gene encoded adjuvants, such as cytokine DNA sequences, have also been studied as potential adjuvants for DNA vaccines. Cytokines are small proteins expressed by leukocytes that modulate the immune system. By delivering cytokines at the site of vaccination, it is possible to specifically tailor the immune response to adequately respond to future challenges. Gene encoded cytokine delivery allows the cytokine to be present at the same time as the antigen, increasing the likelihood that the cytokine can act within the window period where initial immune responses are occurring. Another advantage of delivering cytokines at the site of immunization is the avoidance of systemic exposure, which lowers the possibility of systemic side effects, even in a limited fashion from the vaccine. A vast number of cytokines have been studied as potential adjuvants, including IFN-alpha, GM-CSF, Flt-3 ligand, IL-18, IL-21, IL-15, IFN-gamma, IL-12, and IL-2, in a number of experimental models [30, 31]; importantly, much work still needs to be done in the vaccine field regarding these cytokines as potential adjuvants.

In the context of DNA vaccine gene encoded adjuvants, Interleukin 12 (IL-12) has established an important potency track record for several years and is the most studied cytokine DNA adjuvant in the clinic. IL-12 is a pro-inflammatory cytokine primarily secreted by dendritic cells that connects the innate and adaptive immune response, promoting enhanced Th1 cellular responses. Given its potent Th1 activation, there has been a lot of interest in using IL-12 as an adjuvant in various vaccine platforms, most notably in cancer trials. Early trials where IL-12 protein was delivered systemically resulted in major side effects, limiting potential use. However, local delivery of plasmid encoded IL-12 does not drive systemic toxicity in the clinic [32–34]. Multiple trials have studied pIL-12 as an adjuvant administered as DNA formulated as part of the plasmid vaccine. In this delivery, the IL-12 adjuvanted vaccines have been well tolerated and some of these studies have seen clear immune improvements from the presence of IL-12 adjuvant. A recent study by Kalams et al. is illustrative [32]. In this study the combination of EP + IL-12 drove much improved T cell responses for both CD4 and CD8 immunity. Overall this HVTN study that combined plasmid encoded HIV antigens encoding gag/pol and env + plasmid IL-12 plus Cellectra EP described that the combination approach resulted in overall T cell response rates of 90%, which were similar to combination vaccine studies that required boosting with viral vectors [35, 36]. As another example, a clinical trial that used a multi antigen HIV DNA prime and VSV *Gag* protein boost with increasing doses of plasmid DNA IL-12 [37] found that there were increased CD8 T cell responses in people adjuvanted with plasmid IL-12 compared to those whose vaccine was not adjuvanted. The CD8 responses observed post boost were also enhanced compared to non-adjuvanted groups. As more clinical trials are performed testing IL-12's ability as a potential adjuvant in the DNA setting, especially when combined with EP in additional disease models, we will gain additional insight into the immune activity of these combined approaches.

This initial data has encouraged the study of many additional cytokines, including those whose functions are less well understood, but appear interesting to be investigated as potential adjuvants for DNA vaccines. For example, plasmid encoded

CD40L, which plays a major role in both innate and adaptive immunity, was shown to significantly enhance antigen specific CD8+ T cell responses that were durable at memory timepoints as part of a DNA vaccine cocktail using HPV plasmid antigens [38]. Wise further showed that mice immunized with soluble CD40L had significantly reduced tumor burden in a HPV induced cancer model. Villarreal showed that IL-33, an alarmin that is thought to alert the immune system to different stimuli and tissue damage, was able to act as an immune adjuvant and enhance immune responses in a tuberculosis, LCMV, and cancer model. Villarreal further advanced the field in showing that although IL-33 was traditionally thought to only drive Th2 humoral responses, it has the ability to drive Th1 and CD8+ cellular functions as well [39–42]. Work on interleukin 36 (IL-36), a poorly understood pro-inflammatory cytokine family of the IL-1 superfamily, has begun to shed light on its role in the body and potential as an adjuvant. Preliminary data shows that plasmid encoded IL-36 alpha DNA is able to enhance both CD4+ and CD8+ T cell responses against a HIV Env DNA vaccine (Fig. 16.1). More work is needed to truly tease out the implications of IL-36, given conflicting results of the cytokine from various groups and disease models.

There is a lot of exciting research currently being done in the field to find new potent adjuvants to boost immune responses to vaccines, including research on adjuvant delivery systems, combination studies and plasmid codelivery [43–45]. Adjuvants have the potential to reduce vaccine dose and frequency, overcome immune senescence, and allow for new vaccine targets. As such, it will be critical to further develop this area if we hope to rise to the occasion with the ever-mounting number of EID. A special focus on adjuvants that can be delivered to the skin may prove advantageous, given the large number of antigen presenting cells (APCs) and Langerhans cells found in this tissue as well as the critical immune interactions constantly occurring at this site.

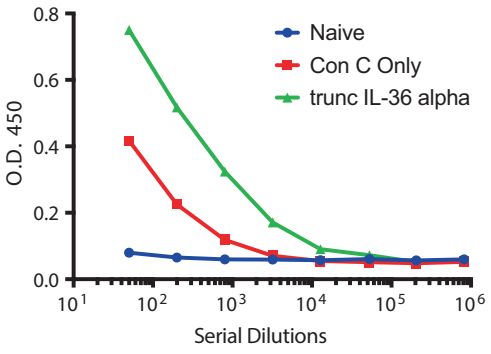

Fig. 16.1 Co-delivery of IL-36 alpha as gene adjuvant enhances humoral responses against HIV-1 DNA vaccine in mice

16.4 The Microbiome and Vaccine Induced Immunity

Over the last 20 years we have come to appreciate that our bodies provide a home to more than ~60 trillion microorganisms, of which at least half are bacterial [46]. These collectively are referred to as the microbiome. It has become apparent that the microbiome is a major important piece of our biology that contributes to our health, and that we could not live easily without this interesting collection of microorganisms. A large body of research has changed some of our thinking about concepts of plasmid delivery in general as it relates to our growing understanding of the microbiome. On average the bacteria that comprise our microbiomes will have a life span of between 12 and 24 h. This means that dying bacteria releasing plasmid and bacterial DNA and thus exposing us to bacterial DNA is a continual natural occurrence. The small amount of DNA that we additionally deliver in a DNA vaccine is likely of little consequence in this grand scheme. In addition to this novel insight into the common exposure to bacterial DNA that live in our bodies constantly, there are additional areas of importance for consideration regarding DNA vaccine induced immunity. One particular area of interest for the vaccine field is the role that the microbiome may play in vaccine-induced immune responses. Data has been coming forth that suggests that the types of bacteria and relative amounts of each type of bacteria may directly impact the efficacy of vaccines. Microbial cells are primarily found in the intestinal tract, as well as the skin, bronchial and genital tract. Studies using germ free mice or those treated with antibiotics to deplete intestinal bacteria have shown defective immune innate responses to infectious diseases including influenza. Upon microbiome restoration, proper immune responses were also restored. These studies also showed the importance of bacteria type. Mice that were colonized with flagellated E. coli mounted the appropriate immune response against influenza A compared to non-flagellated E. coli [47–51]. Given that the microbiome is largely established within the first 6 months of life, around the same time that many vaccines are first administered, additional study of these early colonizers in this context will be important. In a striking study, researchers compared the microbiome of infants from Ghana and the Netherlands, who received the rotavirus vaccine. The Dutch infants were generally able to mount a strong immune response to the vaccine compared to Ghanaian infants. Of the Ghanaian infants that did mount an immune response, their microbiomes were much more similar to the Dutch infants compared to the microbiomes of the nonresponders [52]. The implications of this study suggest that the microbiome may play a significant role in vaccine outcomes, in this case a live attenuated gut vaccine, in different populations. Given that studies suggest that even after microbiome disruption, the same bacteria will reestablish in the intestine, the vaccine field should look in more detail at this issue to learn more about the ways the microbiome can be manipulated for enhanced vaccine immunity. Plasmid encoded adjuvants that can enhance vaccine-induced immune responses and potentially skew the immune response, may represent one potential solution for microbiomes that can negatively impact desired immune outcomes. The biome represented on non-intestinal tissues may also pose unique challenges in vaccination protocols. As the push towards more tolerable and less

invasive vaccine programs such as intradermal vaccine delivery increases, understanding the possible immune interactions between local bacteria on the skin and the immune cells critical in the primary immune response will become increasingly important. Plasmid encoded gene adjuvants may help to enrich the number of antigen presenting cells (APCs) or recruit select populations to the site of vaccination, as a means to overcome potential microbiome interference. One important such study of a synthetic Zika vaccine delivered by the ID route to skin showed that this vaccine was potent and highly consistent from volunteer to volunteer, however more investigation between the microbiome and different vaccines and immunogens is likely to prove important.

As the era of therapeutic vaccine mediated approaches for cancer is well underway, the influence of the microbiome cannot be understated. Clinical studies that evaluated the effectiveness of immune-checkpoint inhibitors that target PD-1 or CTLA-4 found a positive association between the presence of bacteria such as *Akkermansia muciniphila*, *Bifidobacterium* spp., and *Faecalibacterium* and anticancer outcomes [53]. Characterization of gut microbiome of patients with metastatic melanoma treated with anti-PD1 antibodies showed that those who responded to the therapy had a greater abundance of bacteria from the Ruminococcaceae family, of which *Faecalibacterium* is a member. The impact of these families of bacteria on DNA vaccine delivery and potency are worth examining.

In the HPV DNA study previously mentioned, 40% of women treated with the DNA vaccine eliminated the HPV16/18 infection and had complete histopathologic regression compared to only 14.3% in the placebo group. While this represents a major breakthrough as the first therapeutic vaccine to show efficacy against CIN2/3 associated with HPV16/18, there is still a lot of work being done to understand some of the differences between the women who responded and those that did not. Interestingly, some patients were able to regress, but did not clear the underlying infection. As the urogenital tract itself is home to a unique microbiome, a study of the bacteria populations in the patients who cleared and regressed, regressed, or didn't respond is certainly worth investigating (Fig. 16.2).

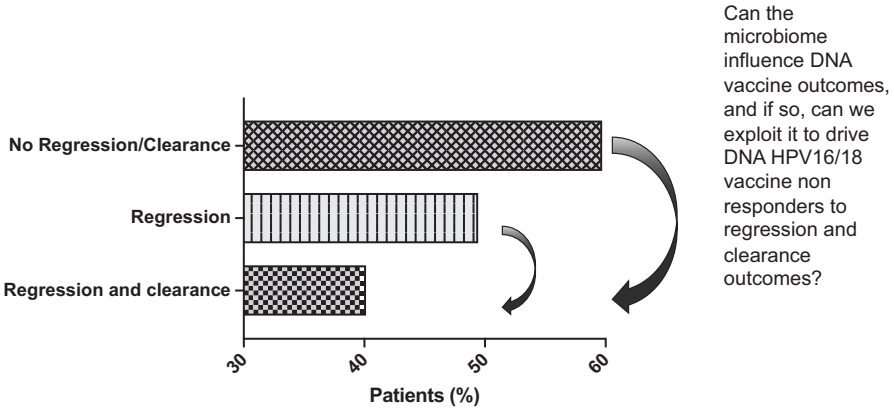

Fig. 16.2 Patient Responses in VGX-3100 DNA HPV16/18 study, vaccinated group (per-protocol)

16.5 Lessons Learned in Rapid Vaccine Development in the Midst of Infectious Outbreaks

Recent global events have highlighted the need for rapid, effective vaccine development for emerging infectious diseases. The World Health Organization (WHO) warned a decade ago that infectious pathogens were emerging and reemerging at rates unseen before. Traditional vaccines have been developed on the scale of years, which is not ideal in the midst of a sudden epidemic, as illustrated by the 2014–2016 Ebola outbreak. In response to this particular outbreak, many groups set out to create therapies and vaccines that could impact these outbreaks, treat those who were infected, or prevent transmission to those that were uninfected. The recent Zika WHO Emergency is a case in point. A team was mobilized to generate a rapid response vaccine to Zika [54–56]. This synthetic DNA vaccine was engineered to generate immunity against the Envelope protein of Zika. It was designed to be delivered into the skin using high concentration formulations of the DNA in a very small volume. As part of the design the vaccine contained sequences encoding the prM region to help with transport and processing of the E antigen. The E antigen is the target of neutralizing antibody responses as it facilitates entry of the Zika virus into target cells. Preclinical experiments performed by the team helped to extend the information about the protective role of anti-Zika antibody responses to the E antigen for animal protection [57–62]. The prME Zika vaccine induced protective levels of antibodies as well as T cells that could protect from Zika infection in laboratory animal models. The vaccine was very potent in non-human primate studies as well as being protective in this species for Zika challenge. The vaccine protected animals from both infection as well as Zika brain and testes pathogenesis. It was moved to the clinic in just over 6 months and became the first vaccine in human clinical testing. The results of this phase I clinical study were recently reported (Tebas et al. [56]). The synthetic prME vaccine-induced rapid seroconversion in greater than 95% of volunteers by two immunizations and 100% seroconversion after three immunizations. Importantly, these antibodies were able to protect immune deficient mice from a lethal Zika virus challenge by passive transfer, suggesting that the antibodies developed through vaccination in vaccine volunteers may be sufficient to protect against subsequent challenge. In addition, T cell responses were induced in most vaccine recipients in this study. More recently, a second DNA vaccine, which was delivered by IM using a ballistic device, was reported. It also generated seroconversion in most vaccinated subjects but used several milligram doses, although the antibody titers induced appear to be lower than those induced by the intradermal electroporation (ID-EP) approach, however more study is important. The use of DNA technologies for outbreak strategies that can be rapidly moved to the clinic appear to be finally establishing an important track record for safety, speed and immune potency.

Importantly, the timeline from concept to clinic for both the Zika DNA vaccines was on the order of months, instead of years, illustrating that these new DNA vaccines appear to be important candidates for rapid outbreak situations (Fig. 16.3).

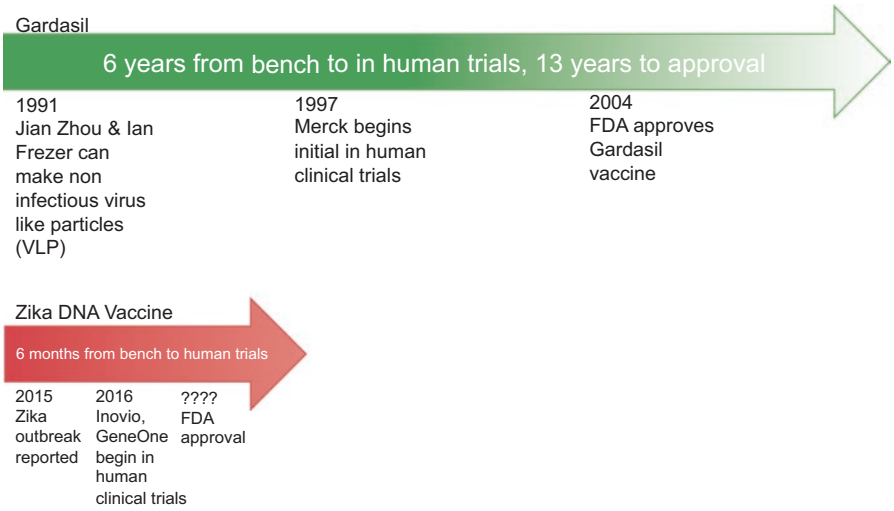

Fig. 16.3 Timeline from bench to in human studies

Not to be forgotten in this discussion is that plasmid DNA's rapid scalability helps to position it as an attractive option in these situations. It is likely that additional studies will provide important performance data in this regard.

16.6 DMAb's: Direct DNA Encoded Antibody Delivery Technology

As illustrated by recent infectious disease outbreaks, there is often a short window to act to prevent massive spreading of disease among vulnerable populations. In these scenarios, additional tools that can be very rapid and further provide population protection are important. The use of direct injection of protective monoclonal antibodies is likely just such a platform. As one major example, during the recent Ebola outbreak a monoclonal antibody cocktail, ZMAPP was deployed to provide some potential relief for Ebola exposed and infected persons, mostly health care workers. This was essentially a post exposure treatment approach aimed at slowing viral progression and allowing the infected person to recover from Ebola. While this delivery may not be long lasting, it may reduce viremia and clinical symptoms until the immune system can kick in or other interventions take place. Some of the main drawbacks to delivering the protein based monoclonal antibodies include high production costs and prolonged development time, lack of temperature stability, as well as short time of expression *in vivo* that likely limits their potential use in many outbreak environments. In addition, traditional passive antibody transfer results in short term expression in the circulation, thereby requiring repeated infusions, further adding to costs and procedures. Sensing a need for a more feasible and cost

friendly alternative, the field began to investigate antibody gene transfer methods that would ultimately allow the body to produce the antibodies without waiting for an immune response to kick in. A majority of the efforts have focused on adenoviral-associated virus-mediated antibody gene transfer. A number of challenge models, including anthrax, RSV, and influenza have shown AAV mediated antibody gene transfer to be effective, if the animals do not have preexisting immunity to the vectors. In the clinic, however there is a high level of pre-existing immunity in the human population to many AAV vectors, which will limit their effectiveness in the clinic. In addition, such vectors would have substantial issues for readministration due to this intrinsic immunity [63–65]. Extensive work is being done to investigate improvements to this promising platform. It should be noted that AAV delivery is a form of gene therapy as delivery can include integration of the delivered AAV vector into the host genome.

The DNA delivery field has also made major progress with DNA plasmid-mediated antibody (DMAb) gene transfer, circumventing many of the issues that the viral vectors face. DNA delivery is transient and does not permanently mark recipients. Accordingly, DNA delivery is more similar to live vaccine delivery which similarly is transient and not gene therapy. This is an advantage for repeating dose studies among others. Many studies have shown that DNA plasmid vectors do not generate anti-vector immunity, allowing for multiple dose administration, making DNA very attractive as a potential platform to encode antibodies. The advancements made in the field discussed earlier including EP and higher concentration formulas, have allowed for greater *in vivo* antibody production, leading to the goal of scaling this platform for clinical studies. This is a very new field for DNA. However, in mouse models of dengue and Chikungunya infection, mice injected with synthetically engineered DMAbs encoding a human neutralizing antibody for either Dengue virus or CHIKV were fully protected against either challenge within just a few days of delivery. These results illustrate the potential strength of the DMAb platform in times where rapid protection is of critical importance.

DMAbs have also been used in tandem with DNA vaccines in order to provide both immediate and long lasting protection [66]. In an elegant study, Muthumani showed that codelivery of a CHIKV DMAb and a CHICK Env DNA vaccine was able to elicit systemic humoral immunity, cell-mediated immunity, and protection *in vivo.* The study also addressed the concern of DMAb antibody interference with vaccine, thereby rendering the two platforms incompatible, by showing that this was not the case and that mice were 100% protected from challenge after codelivery of the two. Administering the DMAbs with a vaccine that will induce a slower but long lasting immune response allows for the best of both worlds. By combining passive immunity through DMAbs and adaptive immunity through vaccine-mediated responses, the DNA platform is able to deliver a full spectrum, robust protective response against infectious agents. Using an influenza model, Elliott was able to show that two novel DMAbs encoding broadly neutralizing antibodies against Influenza A and B respectively, were able to protect mice against lethal influenza challenge, and that the DMAbs delivered coordinately were still able to protect mice against mortality and morbidity, providing a broad protection spectrum against the viruses [67]. Patel demonstrated another powerful advantage of DMAbs

when she reported that two potent DMAbs targeting *Pseudomonas aeruginosa*, including one non-natural bispecific antibody, were indistinguishable from bioprocessed antibody and able to protect against a lethal pneumonia challenge [68]. Recent work has further bolstered the case for DMAb development [69]. Through a series of gene cassette, regimen, and vector optimizations, they were able to enhance DMAb expression, ultimately protecting mice from influenza induced death, but not infection. Importantly, in the same report they were able to protect against Ebola challenge in a mouse model. Together these multiple studies support that DMAb technology represents an important new approach for exploration in a number of infectious disease targets.

16.7 Conclusions and Future Directions

With increased globalization and climate change, novel infectious diseases are an expanding threat to previously unaffected areas, underscoring the need for rapid development of new vaccines. The National Institute of Allergy and Infectious Diseases (NIAID) Biodefense program maintains a record of infectious pathogens and diseases it considers top priorities, which paints a sobering picture of the work in front of us (Fig. 16.4). In addition to new emerging infections, some pathogens previously known can mutate to give rise to new strains that may trigger pandemics. In tackling these pathogens, lessons learned from the Ebola, Zika, and MERS outbreaks can help guide future vaccine programs.

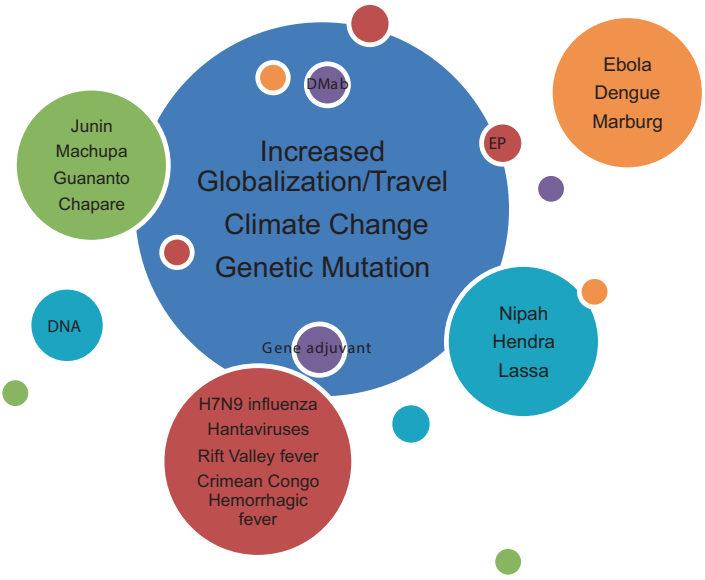

Fig. 16.4 Emerging Infectious diseases according to NIAID

The Synthetic DNA platform has significant potential to contribute to rapidly impacting new outbreaks. Collective advancements to the platform, including higher concentrations of product, improved delivery methods for enhanced EP targeting ID space for example, as well the new DMAb technology, now changed DNA's reputation supporting it as a viable candidate for prophylaxis and therapy options. The inherent properties of plasmid DNA production, including low manufacturing costs, excellent safety profile, rapid scale up potential, high immune response rate of vaccines and short time to clinic, are highly encouraging, especially as the number of efficacy trials is growing. As the platform continues to evolve and target discovery becomes more precise, the promise of this new generation of DNA technologies will be further tested, and grow and be refined. It is exciting to have this important tool available for rapid protection of civilian populations as well as the military.

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Chapter 17
Future Perspectives: Microbiome, Cancer and Therapeutic Promise

Sagarika Banerjee and Erle S. Robertson

Abstract A homeostatic balance exists between a host and its commensal microbes. Disturbance of this homeostasis, a finely tuned system can result in diseases including cancer. Investigating the imbalance of such host-microbiome interactions by comparing the healthy and dysbiotic disease states is important for understanding the pathophysiology of the associated diseases. Evidence is mounting in the field which demonstrates that the dysbiotic microbiome can trigger oncogenic activities and that the microenvironment of different types of cancers allows a distinct microbiome to thrive with the potential for having direct or indirect consequences on the disease progression. An in-depth understanding of the microbial changes and their contribution to disease will provide an informed approach to early diagnosis of these cancers, as well as development of more personalized treatment strategies, and the potential for establishment of normobiosis with microbe-associated cancers.

Keywords Microbiome · Dysbiosis · Microbial biomarker · Cancer · Probiotic · Proteobacteria · Oncovirome

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17.1 Characterization of the Human Microbiome

The most unique organ of the human body, the microbiome is made up of single celled organisms included in the domain Prokaryota, and complex organisms of the domain Eukaryota of living organisms, as well as viruses, which are considered non-living organisms but require a host cell to replicate. The Prokaryota which encompasses the Eubacteria and Archaea bacteria kingdoms, and the Eukaryota which includes the kingdoms Fungi, Protista and Metazoa (Helminths) are included in this complex organization referred to as the microbiome that live in and on our bodies. The term microbiome was coined by Joshua Lederberg to "signify the ecological community of commensal, symbiotic, and pathogenic microorganisms that literally share our body space and have been all but ignored as determinants of health and disease" [1]. All of the genetic material within a microbiota, that is the entire collection of micro-organisms in a specific niche, such as the human gut, is referred as the metagenome of the microbiota in the gut. The number of bacteria and other microbes resident in a healthy human body is either similar to or can even outnumber our own cells [2], and thus, the human microbiome can be referred to as our second genome. We have co-evolved with trillions of these microbes, thus creating a complex, a body habitat-specific, adaptive ecosystem that is constantly tuned with changing host physiology.

Earlier studies to identify the normal microbes colonizing healthy humans by culture technique, highlighted organisms that grow well in the lab environment [3]. Furthermore, the strict anaerobic techniques introduced in the 1970s allowed detection of a higher number of bacterial species from the gut alone [4]. Later, culture independent techniques like DNA sequencing and fluorescence in-situ hybridization (FISH) further allowed direct detection of culture independent microbial DNA from samples [5].

However with the advent of the high-throughput next generation sequencing technologies, characterization of the robust microbiome became possible. It involved shotgun metagenomic sequencing of all of the DNA in a biological samples (human and bacterial) but most commonly involves amplifying, sequencing and analyzing specific regions of bacterial 16S rRNA genes, although other rRNA genes (18S for eukaryotic microbes) or genomic regions (for viruses) can also be used. While some investigators relied upon the 454 pyrosequencing that produce about one million of 400 nucleotide reads per run, others prefer greater sequencing depth offered by whole genome sequencing (WGS) Illumina platforms. In 2012, the Human Microbiome Project (HMP) defined healthy human adult microbiome at multiple body sites in large cohorts [6], using 16S rRNA sequencing and WGS, and showed that each body site has distinct microbial community.

However, while the 16s rRNA sequencing is only limited to bacterial biota detection, and is unable to discriminate between strains or genomic variants, WGS is expensive for screening hundreds of experimental samples and controls in order to establish disease associated microbiome. WGS also contain an overwhelming amount of host DNA sequences that create a huge space for locating pathogenic signatures. Thus, in recent times targeted next-generation sequencing provided the advantage of enriching the microbial signatures from the pool of human genomic sequence [7]. The initial screening of the experimental and control samples by a

pan-pathogen array based system [8], followed by targeted NGS using the probes that screened positive by the array to capture the microbial target directly from the samples [7], provided easier detection and characterization of all the microbes (viruses, bacteria, fungi and parasites) in the samples.

17.2 Microbiome in Health and Disease

The host-microbial interaction plays a major role in shaping the healthy or disease state of the human body [9]. Despite their vital importance in human health and disease, these communities residing within us remain largely understudied. Understanding the broad distinguishing features of a healthy and unhealthy microbiome can provide ways to prevent disease onset and/or improve prognosis.

Evidences from a number of studies have indicated that the mutualistic, resident or transient viruses, bacteria, fungi and parasites in our body generally maintain a careful balance for nutrition, immune-modulation and metabolism that contributes to health; and imbalance leads to microbial dysbiosis, contributing to a range of diseases including cancer [10]. Microbial dysbiosis contributing to the etiology of oral, ovarian, colon, gastric, esophageal, pancreatic, laryngeal, breast and gallbladder carcinomas has been reported [7, 8, 10, 11]. It is likely that immune dysregulation by the dysbiotic microbiome-host interactions can lead to hyper inflammation, dysplasia, proliferation, prevention of apoptosis, and thus to cancer development [12]. Overgrowth of dysbiotic pathobionts could also lead to cellular barrier breach, leading to increased pro-inflammatory signaling and genomic instability [12, 13] (Fig. 17.1). Further, presence of certain oncogenic viruses, bacteria, and parasites in the dysbiotic microbiome could directly cause cellular transformation by encoding certain oncoproteins or effector molecules that leads to genomic instability and dysregulated cell growth [12] (Fig. 17.1).

In 2018, there will be an estimated 1,735,350 new cancer cases diagnosed and 609,640 cancer deaths in the US [14]. Cancer remains the second most common cause of death in the US preceded by heart disease, accounting for nearly one of every four deaths [14]. The different compositions of human microbiome and its contribution to complex diseases like cancer is of interest in recent years, and is still a relatively new field of research. In this regard it has been shown that the differences in the microbiome in an individual can correlate with differences in susceptibility to diseases [15–17]. Additionally, as association with infectious agents is one of the most important contributors to cancer [18], it has been discussed that if infection-associated cancer could be prevented, then there will be a marked reduction in the number of new cancer cases seen worldwide, about 1.5 million less cancer cases in developing countries and 390,000 less cancer cases in developed countries, annually [19]. As for the oncobiome, microbial dysbiosis could be a triggering factor for oncogenesis, or may be, that the tumor micro-environment provides an amiable condition for such oncobiome to thrive. Either way, the oncobiome have been found to be distinctly different from the normal microbiome, and it varied at different body sites [7, 8, 11, 20].

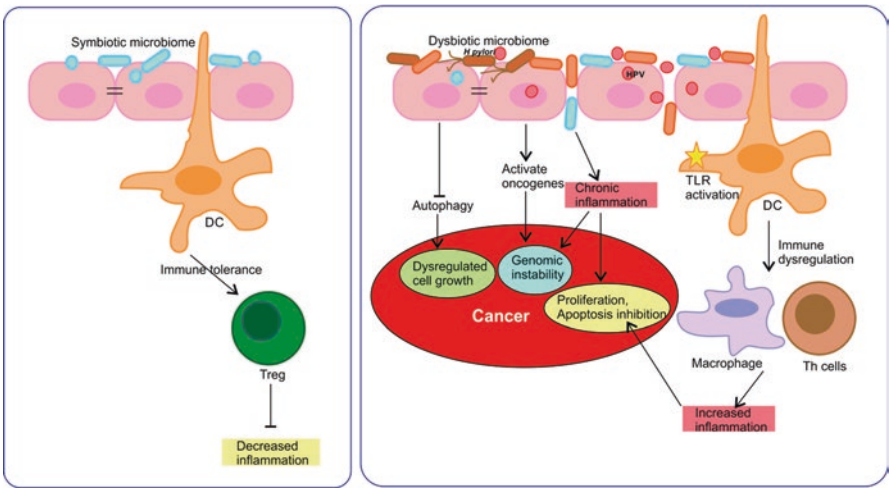

Fig. 17.1 Symbiotic and dysbiotic microbiome in health and cancer. Left panel: a symbiotic microbiome under a functional cell barrier leads to immune tolerance by the development of regulatory T cells (Tregs); Right panel: Overgrowth of dysbiotic pathobionts could breach the cellular barrier, leading to Toll-like receptor (TLR) activation, increased pro-inflammatory signaling and genomic instability. Certain oncogenic viruses, bacteria, and parasites in the dysbiotic microbiome by expressing certain oncoproteins or effector molecules could lead to genomic instability and dysregulated cell growth, thus directly causing cellular transformation

17.2.1 Healthy Microbiome

Low microbial biomass in healthy individuals makes it difficult to characterize the associated microbiota. However, gut, oral cavity, skin and vagina of healthy individual have revealed a robust microbiome association, mostly the bacterial biota than other microbial components [6, 21].

Breast tissues have a unique microbiota, distinct from that found at other body sites [6, 22]. Proteobacteria is the most abundant phylum in breast tissues, unlike in the vagina, oral cavity, bladder, skin, and gastrointestinal tract, where members of this phylum make up only a small proportion of the overall bacterial community [6, 23]. The higher abundance of Proteobacteria and Firmicutes (specifically the class Bacilli) compared with other taxonomic groups in normal breast tissues may be a result of host microbial adaptation to the fatty acid environment in the tissue [23]. GI tract, which has been studied most extensively for associated microbiome [6, 21, 24], shows that a healthy gut microbiome is consistently dominated by bacteria of phyla Bacteroidetes and Firmicutes [6, 21, 24]. Apart from the gut, microbiome associated with other body parts in healthy human has also been studied, and it was found that the microbiome composition is more similar in the same body parts of different individuals, than different body parts in the same individual [6]. Oral cavity, although having complex microbiome as gut tend to be dominated by *Streptococcus* [6]; Skin is being colonized the most by *Corynebacterium,*

Propionibacterium, and *Staphylococcus* [25, 26], with *Propionibacterium acnes* contributing to half of the skin microbiome [27], and the skin-associated archaea *Thaumarchaeota* that make up to 10% of the skin microbiome, particularly in elderly persons and children [28]; Vagina mostly is composed of *Lactobacillus* and *Gardnerella* [29, 30]. Interestingly, a greater abundance of the commensal bacteria *Corynebacterium* and *Kingella* was found to be associated with reduced rate of head and neck cancer [31].

The virome in a healthy microbiome is understudied as sequencing of metagenomes has often ignored the viruses. However, metagenomic studies of microbiota at various tissue sites have revealed that many of the viruses associated with healthy human tissues are bacteriophages [32–34]. It is also estimated that an individual healthy human harbors >10 permanent chronic systemic viral infections that include herpesviruses, polyomaviruses, anelloviruses, circoviruses, adenoviruses, papillomaviruses, endogenous retroviruses, and hepatitis viruses [35]. Merkel cell polyomavirus, Polyomavirus HPyV7, Human papillomavirus, endogenous RD114 retrovirus, and members of Circoviridae have been found abundant amongst normal skin flora along with certain phage families (Myoviridae and Siphoviridae) [25, 32, 36]. Among the normal human oral virome, the vast majority of the human salivary viruses identified were bacteriophages for *Veillonella*, *Streptococcus* and *Megasphaera* [32, 34], and it also included low risk HPVs (HPV 6, 11) and Herpesviridae (EBV, HSV1) [37–41]. Ninety percent of the normal gut virome comprises mostly of intestinal bacteriophages [42]. Mostly, the gut phages in healthy adults belong to the order Caudovirales with double-stranded DNA (Podoviridae, Siphoviridae and Myoviridae) or single-stranded DNA viruses from the families Microviridae and Inoviridae, most of which are temperate ones, in which phages integrate into host chromosomes or exist as quiescent episomal elements at the expense of lytic replication [43–45]. This is important for genetic exchange between bacterial hosts, alteration of host phenotypes via lysogenic conversion, which in turn impacts on bacterial host fitness as well as human gut microbial dynamics [44, 45]. Other than lysogenic phages, the GI virome also comprised of Enterovirus, Rotavirus, Calicivirus, Astrovirus and Adenovirus, Kobuvirus (Aichi virus), Parechovirus, Cardiovirus (Saffold virus), Anellovirus, Picobirnavirus, Polyomaviruses (BK, JC and SV40 viruses) and large viruses of family Mimiviridae, Mamaviridae, Marseilleviridae [32, 45–47].

The study of the eukaryotic component of the human microbiome is lagging compared to the bacterial communities. Among the healthy individuals, the mycobiome constitutes the 'rare biosphere' (<0.1%) of the entire microbiome [48], comprising mainly of *Candida*, *Malassezia* and *Saccharomyces* [48, 49]. Culture dependent and independent techniques have revealed different mycobial generas associated with different niche of healthy individuals. The healthy oral mycobiome included genera of *Candida, Cryptococcus, Cladosporium, Aureobasidium, Aspergillus, Fusarium, Malassezia, Epicoccum* along with abundant non-culturable fungi and environmental fungi [48–51]; A healthy gut mycobiome is predominated by the fungal genera *Candida* and *Saccharomyces* [48, 52], healthy skin mycobiota included commensal fungi, that included *Malassezia, Penicillium,*

Aspergillus, Alternaria, Candida, Rhodotorula, Cladosporium [53, 54]; The commonly detected healthy vaginal mycobiota included *Candida, Saccharomyces, Aspergillus, Alternaria,* and *Cladosporium* [55–57]. Although lungs are exposed to the oral microbiota, there is not much evidence of commensal lung mycobiome [58]. However, the common fungi in lungs include *Aspergillus* sp. and *Scedosporium* sp. [59].

Apart from the mycobiome, human associated protists and helminths constitute the other part of the eukaryome. Although, historically, any protist or helminth in human was considered parasitic and/or pathogenic, recent studies have shown the presence of such lower eukaryotes among the normal human microbiome. For example, *Blastocystis* and *Dientamoeba* were detected frequently in the GI of healthy individuals [60–62]; *Entamoeba*, *Trichomonas* are known healthy oral parasites [63]; *Demodex* are known to inhabit human skin [64].

17.3 Dysbiotic Microbiome and Cancer

The unbalanced microbial profile, or, dysbiosis often has been correlated with the genesis and evolution of complex diseases such as cancer [20]. Either a dysbiotic microbial community with pro-carcinogenic features remodels the microbiome as a whole to drive pro-inflammatory responses and epithelial cell transformation, leading to cancer, and/or, the "microbial drivers", initiate transformation by inducing epithelial DNA damage and tumorigenesis, in turn promoting the proliferation of passenger micro-organisms that have a growth advantage in the tumoral microenvironment [65, 66]. While viruses are known for their direct cellular transforming ability, either through expression of certain viral oncogenes or, through integration of its genome into host chromosomes causing genomic instability and altered expression of cellular proto-oncogenes, tumor suppressors; they can also function as indirect transforming agents through virus-induced chronic infection and inflammation [67]. The role of non-viral microbiome in driving oncogenesis is understudied, especially that for fungi and parasites. Recent studies show that a balanced bacterial microbiome although it may be involved in prevention of tumor development, but when altered (dysbiosis) may participate in carcinogenesis [68]. Bacterial mechanisms implicated in carcinogenesis include directly DNA-damaging toxin secretion, induction of chronic inflammation and suppression of immune cell activation [69, 70]. For example, *Chlamydia* is known to contribute to cancer by inhibiting apoptosis, inducing DNA damage response and increasing susceptibility to other infections [71]. Prostate cancer, the leading cancer in males in the United States [2] has often been preceded by inflammatory responses in the prostate [72, 73]. The dysbiotic microorganisms in the prostate can enhance the inflammatory responses and contribute toward cancer development [2, 74–80]. Significant perturbations in the microbiome, resulting in a specific tumor microbiome signature have been reported for different cancers.

17.4 The Dysbiotic Virome in Cancer

Several studies have shown an association of oncogenic DNA viruses with different cancers, mainly the high risk Human Papillomaviruses (HPV), Polyomaviruses and Human herpesviruses (HHV); JC Polyomavirus (JCV), HHV4, HHV8, HHV5, HHV6a, HHV6b, high (HPV16, HPV18) and low risk HPV associations with ovarian cancer [11, 71, 81, 82]; HPV-18, JCV, BK polyomavirus (BKV), Human cytomegalovirus (HCMV/HHV5), Epstein-Barr virus (EBV) with prostate cancers [74, 75, 77, 78, 80, 83–85]; HCMV, EBV, HPV16, HPV-31, HPV-45, HPV-52, HPV-6, HPV-66, Simian virus 40 (SV40), Merkel Cell Polyomavirus (MCPV) and JCV with breast cancer [8, 86–91]; MCPV, HHV8 and HPVs in skin cancer [92]; Dominant detection of oncogenic HPV16 in 35–98% of the oral cancer samples [7, 93, 94] and in cervical cancers [95, 96], while low risk HPVs (HPV2, HPV6b, HPV1) detected less commonly in these cancers [7, 94, 95]. Several studies indicated that a dysbiotic bacterial microbiome could be involved in HPV persistence in those cancers [96]. Additional DNA viral signatures detected in oral cancers included Herpesviridae, Poxviridae and Polyomaviridae [7, 97]; Epstein-Barr Virus (EBV) was detected in 40–60% of oral cancer squamous cell carcinoma (OCSCCs) in some studies [98, 99], and in the majority of the OCSCCs in another study [100]. The higher percentage of EBV positivity was seen to correlate with the increasing grade of OCSCC [101]. Specific Poxviridae signatures of Yaba Monkey tumor virus was seen to be associated with ovarian cancers [11]. Certain members of Herpesviridae (EBV, KSHV, HCMV), and HPVs were found to be involved in benign and malignant proliferative diseases of the gastrointestinal tract [102, 103], while *Helicobacter* phages, KHP30 and KHP40 were considered to contribute to the bacterial evolution that may contribute indirectly to the bacterial pathogenesis [104]. The detections of HPV16, HPV18, EBV, KSHV and Torque Teno Virus (TTV) are often associated with lung cancer development [105–109]. In fact the KSHV latent transcripts detected in lung neoplasm were human homologous oncoproteins (viral cyclin-D), inflammatory cytokines (viral IL-6), and inhibitors of apoptotic pathways (viral FLIP and viral Bcl-2) [108], and thus could play a role in the oncogenesis.

RNA viruses can also contribute to the oncovirome. The association of Retroviruses with cancer has been seen in multiple studies. Retroviral signature is seen to be associated with oral cancers [7]. Mouse mammary tumor virus-like DNA were detected in ovarian cancers [11, 110, 111], breast cancer [8] and in the 36% of prostate cancers [110]. However, the association of the endogenous retrovirus, Xenotropic murine leukemia related virus (XMRV) in familial prostate cancer patients have been controversial [76, 112].

Viruses known to be direct transforming agents, either express certain proteins that control host cell death and proliferation, or, it integrates certain viral genes or its genome in the host chromosomes resulting in deregulation in the expression of cellular oncogenes or tumor suppressor genes [113]. HHV-6A and HHV-6B viral genome integration seen, mostly at the telomeric/sub-telomeric region of several

host chromosomes in ovarian cancer cells [11, 114, 115], and at a number of significant host genomic sites that play an important role in regulating cell proliferation and apoptosis, which may further relate to the genesis of ovarian cancer [11].

The distribution of the integration sites for high risk HPV16 in the host chromosomes and the association of such integrations in regulating cellular cancer-related genes have been reported [7, 116]. The HPV16 genomic insertion were seen mostly at the intronic regions [7, 117, 118], and at the region around the polyA sequence of the E5 gene in the cancer cells [7, 116]. JC Polyomavirus Large T antigen, VP1, VP2 and VP3 sequence insertions have been reported at the intronic regions of certain genes whose de-regulation is associated with numerous cancers [7].

It has been reported earlier that 100% of HPV18 positive cancers showed viral integrations [119, 120]. Although how certain viral genomic DNA integrates at random sites on the host chromosomes is unknown, many nuclear viruses are able to occasionally integrate at the chromosomal fragile sites that are formed due to DNA damage, oxidative stress etc. Also many large DNA viruses have cellular homologous genes [121], and how the viruses acquire such genes remain elusive.

17.5 The Dysbiotic Bacterial Microbiome in Cancer

The pro-cancerous effect of bacterial microbiome dysbiosis has been studied extensively. Although, higher abundance of Proteobacteria has been associated with dysbiosis related diseases including cancer [7, 11, 122–124], the dysbiotic microbiome varied at different body sites. The dysbiotic bacterial microbiome may be pro-inflammatory, may affect normal metabolism and/or, can cause DNA damage, thus leading to host cell transformation [10]. Very little is known about bacterial DNA integrations into the host genome, a consequence of which could be the alteration of host gene expressions, ultimately leading to carcinogenesis [125], although such events are known for viruses. Bacterial DNA integrations into host genomes through RNA intermediates occur more frequently in tumors than in normal samples [125]. Random bacterial DNA integrations of *Acinetobacter* DNA in the human mitochondrial genome, *Pseudomonas* DNA integration in the 5′ and 3′ UTR of 4 proto-oncogenes showing increased transcription along with its conversion to oncogene [125] provides additional insights into the possible role of the dysbiotic bacterial microbiome. Numerous bacterial genomic insertions have been detected, especially in the exons of certain host genes of oral squamous carcinoma tissues: like the tumor suppressors ADAMTS1 (with *Mycobacterium* genomic element integrations), RASSF5 (with *Aeromonas* genomic insertions), and the SMURF2 gene (with *Escherichia coli* genomic insertions), the chromatin re-modelling gene SRCAP (with *Sphingomonas* genomic insertions) and the proto-oncogene WNT3 (with *Bordetella* genomic insertions) [7]. Numerous bacterial DNA insertional sites at the exonic, intronic, UTR, ncRNA, upstream and downstream of host genes involved in many cellular functions have been suggested [7, 11].

Many studies have been carried out to look for bacterial flora associated with oral cancer [7, 126–130]. There is currently no consensus among studies on the dysbiotic nature of the bacterial microbiome in oral cancers. Thus, it is not possible to understand if any bacterial dysbiosis identified in the oral cancers involve the aetiology of cancer, or is just a consequence of it. The significant bacterial signature specific to oral cancer was the increased detection of Proteobacteria observed in the cancers far more than matched (non-cancerous oral tissue from the same patient) and healthy non-matched controls [7, 130]. Although the bacterial flora at the phylum level was not significantly different, the bacterial genuses detected within the phylum were noted to be significantly different between cancer and controls. One study showed a reduction in the abundance of Firmicutes (*Streptococcus*) and Actinobacteria (*Rothia*), and an increase in abundance of Fusobacteria (*Fusobacterium*), when compared with their respective matched-controls, but a greater abundance of Bacteroidetes (*Prevotella*) in oral cancer patients when compared to healthy non-matched controls [129]. While another study showed a slight decrease in the abundance of Firmicutes, and not much change for the Actinobacteria in oral cancer samples when compared to matched-controls, a drastic reduction in the abundance of Bacteroidetes were seen in both cancer and matched controls when compared to non-matched controls [130]. Some other study detected Firmicutes and Bacteroidetes in oral cancer patients [128]. Overall, the bacterial genuses associated with oral cancer in different studies included *Veillonella*, *Fusobacterium*, *Prevotella*, *Porphyromonas*, *Actinomyces*, *Clostridium*, *Haemophilus*, *Enterobacteriaceae*, *Streptococcus*, *Clostridium, Escherichia, Brevundimonas, Comamonas, Alcaligenes, Caulobacter, Cardiobacterium, Plesiomonas, Serratia, Edwardsiella, Haemophilus, Frateuria, Rothia, Gemella, Johnsonella, Capnocytophaga and Peptoniphilus* [7, 127, 128, 130, 131].

Like normal breast tissues, Proteobacteria and Firmicutes, were also the predominant microbiome in the breast cancer tissues. *Brevundimonas* genus was detected in the breast cancers as the most prevalent among the Proteobacterias [8]. The *Mobiluncus*, *Prevotella*, *Rothia* were the other predominant bacterial genera detected in the breast cancers [8].

Prostate cancer often being preceded by inflammation, an over-representation of *Chlamydia trachomatis* and *Propionibacterium acnes* has been known for an increased risk for prostate cancer development due to their pro-inflammatory host responses [132, 133]. Chronic *Mycoplasma* infection was seen to have a causal role for prostate cancer development as infected benign prostatic hyperplasia (BPH) cells lead to cancer [134]. There have been reports of *H. pylori* in the prostatic tissue of both patients with BPH and prostate cancer [135, 136].

It was seen that women with cervical cancer have a more diverse *Lactobacillus*-depleted vaginal microbiome, compared with healthy women, and the dysbiotic microbiome most likely is involved in HPV persistence [96].

Few studies reported the dysbiotic nature of the bacterial microbiome in ovarian cancers [11, 71, 137, 138]. Ovarian cancer microbiome comprises of *Brucella*, *Chlamydia*, *Mycoplasma* [71, 137, 138]. Another study showed abundance of *Pediococcus, Burkholderia*, *Sphingomonas*, *Enterococcus*, *Staphylococcus*,

Treponema, *Francisella, Shewanella* detected in majority of ovarian cancer samples screened [11].

The predominant bacterial microbiome associated with different types of cancers [139–156] may include increased abundance of otherwise commensals or pathogenic bacteria. The association of *Streptococcus*, *Staphylococcus*, *Bacillus*, *Mycoplasma*, *Chlamydophila Granulicatella, Abiotrophia Pneumococcus, Mycobacteria* in lung cancer [140, 144, 145, 147, 148, 151, 155] has been well documented. In fact, one preliminary study showed that sputum dysbiosis associated with lung cancer correlated with an increased relative abundance of *Granulicatella, Abiotrophia and Streptococcus*. Also, a dysbiotic vaginal flora with an increased diversity of vaginal microbiota (for example, *Sneathia, Fusobacterium*), combined with reduced relative abundance of *Lactobacillus* is involved in HPV acquisition and persistence and the development of cervical pre-cancer and cancer [96]. Other predominant bacterial genera associated with cancers include *Pediococcus* in pancreatic cancer [145, 150]; *Staphylococcus, Mycoplasma and Chryseobacterium* in breast cancer [141, 145, 154]; *Staphylococcus aureus* in squamous cell carcinoma of skin [157]; *Fusobacterium* and *Prevotella* in oral cancer [145, 146]; *Treponema* and *Streptococcus* in oesophageal cancer [152]; *Salmonella* in gall bladder cancer [145, 153]; *Chlamydia* in Pulmonary Mucosa-Associated lymphoid tissue lymphoma [143, 145]; *Streptococcus*, *Fusobacterium*, *Escherichia* and *Mycoplasma* in colorectal cancer [139, 142, 145, 149, 154]; *Citrobacter, Clostridium, Lactobacillus, Achromobacter* and *Rhodococcus*, otherwise intestinal mucosa commensals found to be abundant in gastric cancers [158] along with *Campylobacter, Streptococcus* and *Helicobacter pylori* [159, 160].

17.6 The Dysbiotic Fungal Mycobiome in Cancer

Chronic chromoblastomycosis in seven patients caused by Fonsecaea has been reported to lead to squamous cell carcinoma [175]. Fungal infections in cancer patients are common. The abundant detection of yeasts in the cancer cases is expected, given the opportunistic nature of these fungi. Among the fungi, yeasts like *Candida, Geotrichum, Rhodotorula, Trichosporon, Pneumocystis* and fungi causing Mucormycosis, Aspergillosis (cutaneous infections) as well dermatophytes like *Epidermophyton* and *Trichophyton* are commonly known to be associated with cancers [7, 8, 11, 162–167]. *Candida* infection associated with oral leucoplakias showed higher rate of oral malignant transformation [168–171]; Dysbiotic vaginal flora with increased *Candida* infection is often associated with high risk HPV18 infection [171]; *Rhodotorula, Geotrichum*, *Pneumocystis* seen specifically only in oral cancer patients [7, 172, 173]; *Cladosporium* detected in abundance in the ovarian cancers [11] and *Phoma, Candida* in Colorectal/GI cancer [174]. High incidence of microsporidia like *Encephalitozoon* and the fungi *Fonsecaea* in cancers is common [7, 8, 175, 176]. Particularly, *Fonsecaea* infection is seen to predispose squamous cell carcinoma development [177], and also has been reported to be

present in the oral and breast cancers and not in healthy controls [7, 8]. Another microsporidia, *Pleistophora* is seen to be associated with breast, oral and ovarian cancers [7, 8, 11, 176].

Fungi of low pathogenicity like *Malassezia* and *Absidia*, along with the dermatatious aetiologic agents of chromoblastomycosis, *Phialophora* and *Cladophialophora* seen to be associated significantly with the oral and ovarian cancers [7, 11] can cause significant infection and morbidity in cancer patients [178].

How an altered fungal microbiome affects the course of carcinogenesis is to date mostly unexplored. Fungi, could act as a primary pathogen, and weaken the host immune system (through fungal toxins) by releasing free radicals, that can damage DNA, or, it may act as an opportunistic pathogen, causing illness by taking advantage of immunocompromised hosts [179]. The 18S rRNA genomic integration of fungal genomic fragments in oral cancer host chromosome has been reported [7], which is intriguing, with fragments of *Pleistophora, Geotrichum, Phialophora* and *Rhodotorula* seen to be integrated at the intronic and upstream of certain tumor suppressors, and other host genes that are associated with cancer development [7]. However, whether such integrations affect the host gene expressions and contribute towards oncogenesis is speculative.

17.7 The Dysbiotic Parasitic Microbiome in Cancer

Among the parasitic protists, the association of some Apicomplexan and Flagellate species with neoplastic changes in the host tissues is known [180]. It was demonstrated recently that the Apicomplexan *Cryptosporidium parvum* can generate invasive cancer in gastrointestinal and biliary epithelia of SCID mice [180, 181], and *Theileria* was shown to be able of inducing a reversible, parasite-dependent transformation of leukocytes [182]. Interestingly, some of the intracellular protists (*Leishmania, Trypanosoma, Cryptosporidium, Toxoplasma, Plasmodium, Theileria*) are known to induce apoptosis inhibition [183], an effect that could be a significant step in the progression to malignancy [184]. Thus, some parasitic worms of the human body, as well as parasites acquired by ingesting raw fish and meat can increase the risk of developing certain cancers.

DNA of intestinal parasites, *Hymenolepis*, *Centrocestus* and *Trichinella* is detected in the oral cancer samples but not in the control samples [7]. There have been reports on the association of intestinal parasites like *Trichinella*, *Trichuris, and Schistosoma* with different cancers like prostate, bladder, colorectal, breast, ovarian and oral cancers [7, 8, 11, 180, 185–189]. Epithelial dysregulation and hyper proliferation during chronic infection of *Trichuris* [190] has also been reported, which potentially could promote tumorigenesis. The association of other parasites like *Echinococcus, Strongyloides, Leishmania, Ascaris, Trichomona*s to cancer has also been reported [7, 8, 11, 191, 192]. Vaginal dysbiosis with increased infection by *Trichomonas vaginalis* is seen to be associated with higher prevalence of high risk HPV infections [171]. DNA of the zoonotic parasite *Dipylidium* was detected in

ovarian cancers [11]. There is also evidence of parasite sequence integration in host genome. For, example sequences of the parasite *Trypanosoma cruzi* were integrated into human somatic cell genomes, disrupting host genes [193]. There is report of parasite sequence insertions in the host chromosomes of oral cancer patients [7]: *Strongyloides, Contracaecum, Trichinella, Echinococcus* and *Prosthodendrium* genomic sequence in the proximity of certain proto-oncogenes, tumor suppressors and miRNAs have been reported, which may alter expression and further contribute to oncogenesis [7].

17.8 Distinct Microbiome Signatures as a Diagnostic and Prognostic Marker for Particular Cancer

Due to variations between individuals, use of the microbiome to improve cancer diagnostic and treatment becomes a challenging task. However, with the recent upsurge in studies related to microbial dysbiosis in cancers, we are getting closer to identifying a distinct microbial signature pattern for different cancer types. This will allow for a deeper understanding of its role in the oncogenic process and so provide guidance for therapeutic decisions, treatment monitoring and prediction of response. We have seen that various cancers have a robust and varied microbiome with aspects that are unique to each type as well as shared components (Table 17.1). A distinct microbial signature for a particular cancer type may act as a diagnostic marker. For the microbes to be considered disease-specific biomarkers or, microbial biomarkers, they must be associated directly with the condition in question, but not necessarily the cause [201]. Thus, certain microbial signatures consisting primarily of HPV16 among virome; bacterial signatures of certain Proteobacterias (*Escherichia, Brevundimonas, Comamonas, Alcaligenes, Caulobacter, Cardiobacterium, Plesiomonas, Serratia, Edwardsiella, Haemophilus, Frateuria*), *Actinobacteria* (*Rothia*) and Bacteroidetes (*Peptoniphilus*); fungal signatures of *Rhodotorula, Geotrichum, Pneumocystis* and parasitic signatures of *Hymenolepis, Centrocestus, Trichinella* associated only with the oral cancer tissues and not with the controls could be used as diagnostic markers of such cancers [7]. A significant association of a human variant or family member of the Yaba Monkey tumor virus like sequences identified in ovarian cancers, along with Human Herpesvirus 6 (HHV6) and HPV18 signatures could be crucial for detection of ovarian cancers [11]. A distinct diagnostic microbiome signature could differentiate between colorectal cancer and control group [202], and in fact germ free status or administration of antibiotics showed reduction of number of colorectal tumors in experimental models [203, 204]. Again, probiotics shifted the gut microbiome towards beneficial bacteria like *Prevotella*, and *Oscillibacter* which are producers of anti-inflammatory metabolites that was shown to repress hepatocellular carcinoma in mice [205].

Thus the initial maps of microbial associations with different cancers which were not seen in the controls can serve as potential diagnostic tools for early detection of

Table 17.1 Microbiome in health and cancer

Body sites	Healthy	Cancer
Oral	**Virus**: Bacteriophages for *Veillonella*, *Streptococcus* and *Megasphaera* [34], Herpesviridae (EBV, HSV1) [32, 38, 40, 41], HPV 6, 11 (low risk HPVs) [37, 39] **Bacteria**: *Streptococcus* [6] *Prevotella, Moraxella, Actinomyces* [2], *Corynebacterium, Kingella* [31] **Fungi**: *Candida, Cladosporium*, *Aureobasidium*, *Saccharomycetes*, *Aspergillus*, *Fusarium*, *Cryptococcus*, *Malassezia, Epicoccum* [48–51] **Parasite**: *Entamoeba*, *Trichomonas* [63]	**Virus**: HPV16 (high risk HPV), Herpesviridae (EBV), Poxviridae, Polyomaviridae [7, 97–101] **Bacteria**: *Exiguobacterium*, *Prevotella*, *Staphylococcus, Veillonella, Micrococcus, Capnocytophaga* [126], *Fusobacterium, Porphyromonas, Clostridium, Streptococcus, Haemophilus* [194], *Eubacterium, Leptotrichia* [206], *Escherichia, Rothia, Peptoniphilus, Brevundimonas, Comamonas, Alcaligenes, Caulobacter, Cardiobacterium, Plesiomonas, Serratia, Edwardsiella, Haemophilus, Frateuria* [7] **Fungi**: *Candida, Rhodotorula, Geotrichum*, *Pneumocystis, Pleistophora, Malassezia, Absidia Phialophora, Cladophialophora* [7, 168–173] **Parasite**: *Hymenolepis*, *Centrocestus, Trichinella* [7]
Skin	**Virus**: Polyomavirus: HPyV6, HPyV7, Merkel cell polyomavirus, Human papillomavirus (β and γ), endogenous RD114 retrovirus, Circoviridae [25, 32, 36] **Bacteria**: *Corynebacterium, Propionibacterium, and Staphylococcus epidermis* [25, 26], *Thaumarchaeota* [28], *Acinetobacter*, *Micrococci* [27] **Fungi**: *Malassezia, Penicillium Aspergillus, Alternaria, Candia, Rhodotorula, Cladosporium, Epicoccum, Trichophyton* [25, 53, 54] **Parasite**: *Demodex* [64]	**Virus**: HPV, HHV8, Merkel cell polyomavirus [92] **Fungus**: *Fonsecaea* [177] **Bacteria:** *Staphylococcus aureus* [157]

(continued)

Table 17.1 (continued)

Body sites	Healthy	Cancer
Gastro intestine	**Viruses**: Bacteriophages, Enterovirus (Poliovirus, Echovirus, Coxsackievirus), Rotavirus, Calicivirus, Astrovirus, Adenovirus, Kobuvirus (Aichi virus), Parechovirus and Cardiovirus (Saffold virus), Anellovirus, Picobirnavirus, Mimiviridae, Mamaviridae, Marseilleviridae, BK, JC and SV40 [32, 45–47] **Fungi**: *Candida, Saccharomyces, Trichosporon, Cladosporium* [5, 52, 195, 196] **Bacteria**: Prevotella, Ruminococcus, Methanobrevibacter [52] **Parasites**: *Blastocystis, Dientamoeba* [60–62]	**Virus**: Herpesviridae (EBV, KSHV, HCMV), HPV [102, 103], Helicobacter phages KHP30 and KHP40 [104] **Bacteria**: *Phyllobacterium, Achromobacter, Citrobacter, Lactobacillus, Clostridium, Rhodococcus* [158], *Campylobacter, Streptococcus, Helicobacter pylori* [159, 160] **Fungi**: *Phoma, Candida* [174] **Parasite**: *Schistosoma* [180]
Lungs	**Virus**: Paramyxoviridae, Orthomyxoviridae, Picornaviridae, Rhinovirus, respiratory syncytial virus, Adenovirus, Anelloviride **Fungi**: *Aspergillus, Scedosporium* [59]	**Virus**: Herpesviridae (EBV, KSHV) [106–108], Papillomaviridae (HPV16, HPV18) [109], Torque teno virus [105] **Bacteria**: *Granulicatella, Abiotrophia, Streptococcus, Mycobacterium* [144, 147, 155], Chlamydia pneumoniae [148], Pneumococcus [151]
Liver	Data not available	**Virus**: Hepatitis C virus [197] **Bacteria**: *Helicobacter pylori* [197]
Breast	Bacteria: Mostly Proteobacteria, and then Firmicutes; *Bacillus, Acinetobacter, Pseudomonas, Staphylococcus, Propionibacterium, Gammaproteobacteria, Listeria, Micrococcus, Staphylococcus, Streptococcus* [23].	**Viruses**: HCMV, EBV, HPV16, HPV-31, HPV-45, HPV-52, HPV-6, HPV-66, Simian virus 40 (SV40), JCV, Mouse mammary tumor virus [8, 86–91] **Bacteria**: *Brevundimonas*, *Mobiluncus*, *Prevotella*, *Rothia* [8], *Escherichia* [23], *Staphylococcus, Mycoplasma and Chryseobacterium* [141, 145, 154] **Fungi**: *Pleistophora* [8] Parasite: *Trichinella*, *Trichuris, Toxocara, Leishmania* [8, 187]

(continued)

Table 17.1 (continued)

Body sites	Healthy	Cancer
Ovarian	Data not available	**Viruses**: JC Polyoma, HHV4, HHV8, HHV5, HHV6a, HHV6b, HPV16, HPV18, Mouse mammary tumor virus [11, 71, 81, 82, 110, 111] **Bacteria**: *Pediococcus, Burkholderia*, *Sphingomonas*, *Enterococcus*, *Staphylococcus*, *Treponema*, *Francisella, Shewanella* [11] **Fungi**: *Cladosporium, Pleistophora, Malassezia, Absidia Phialophora, Cladophialophora* [11] **Parasite**: *Dipylidium, Strongyloides, Trichuris, Trichinella, Leishmania, Dipylidium* [11]
Vagina	**Bacteria**: *Lactobacillus* [29, 30] **Fungi**: *Candida, Saccharomyces, Aspergillus, Alternaria, and Cladosporium* [55–57]	**Virus**: HPV [96] **Bacteria**: *Gardnerella, Prevotella, Clostridiales, Bacteroides, Sneathia, Fusobacterium* [96] **Fungi**: *Candida* [171] **Parasite**: *Trichomonas vaginalis* [171]
Prostate	**Bacteria**: Actinobacterium, Propionibacterium acnes, Chlamydia, Mycobacterium, Trichomonas [198, 199]	**Viruses**: HPV-18, JCV, BK polyomavirus (BKV), Human cytomegalovirus (HCMV/HHV8), Epstein-Barr virus (EBV), Mouse mammary tumor virus [74, 75, 77, 78, 80, 83–85, 110] **Bacteria**: *Escherichia coli, Pseudomonas, Propionibacterium acnes, Corynebacterium, Staphylococcus* [200]

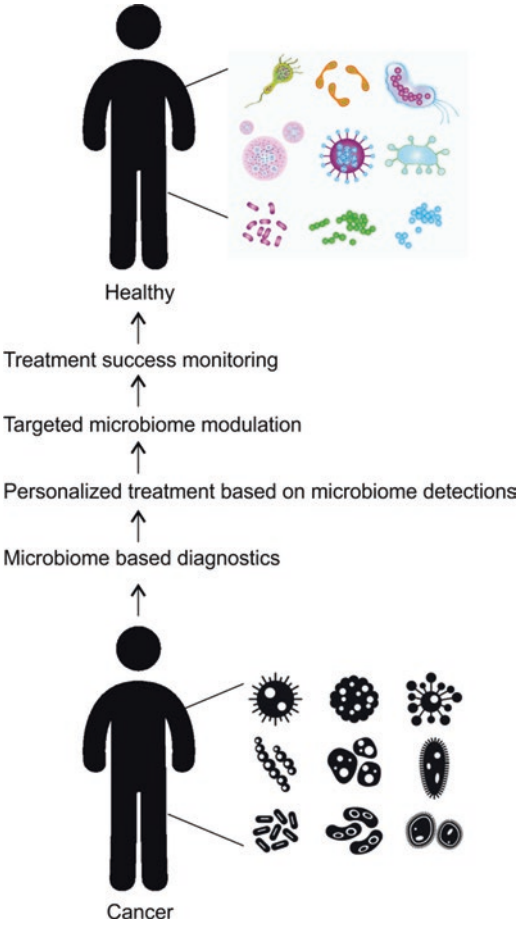

Fig. 17.2 Cancer associated microbiome as a diagnostic and prognostic marker. A cancer patient's microbiome markers can be used for diagnosis and based on the patient's microbiome, a microbiome-based therapeutic approach can be developed. By using precision probiotics, prebiotics, targeted antibiotics and phages, the establishment of normobiosis can be achieved

each of those cancer types. Overall, these studies indicate that the microbiome field is slowly but definitely approaching and realizing its potential for utility towards clinical applications (Fig. 17.2). First, microbiome markers can be used for diagnosis (and potentially prognosis) of disease. Second, analysis of patient microbiota could predict the outcome of treatment options. Third, based on the patient's microbiome, a personalized interventional strategy can be developed, be it based on the administrations of specific microbial cocktails ('precision probiotics'), targeted microbial nutrients ('precision prebiotics'), personalized dietary interventions or targeted antibiotics and phages. Finally, treatment success and establishment of normobiosis can be monitored to determine individuals who may be prone to have relapses as their signatures change. The multiple aspects of this microbiome-based therapeutic approach are nearing clinical implementation and is increasingly becoming a true translational discipline.

17.9 Conclusion

Thus the human microbiome is comprised of mutualistic, pathogenic, transient and residential viruses and microorganisms. Many recent studies have suggested that the body's microbiome dramatically affects health, where perturbation of the microbiome leads to altered physiology and pathology, including cancer. However, the reverse may also be true, that different human diseases create disease microenvironments amenable to the persistence of a differential microbiome, with or without a direct effect of the establishment or progression of the disease. Such differential microbiomes could be specific to each such disease, and thus may provide insights for diagnosis, prognosis, prevention and the development of treatments for microbe-associated cancers.

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