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Viruses and Human Cancer: From Detection to Causality

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Abstract

The study of cancer is incomplete without taking into consideration of tumorigenic viruses. Initially, searches for human cancer viruses were fruitless despite an expansion of our knowledge in the same period concerning acute-transforming retroviruses in animals. However, over the last 40 years, we have witnessed rapid progress in the tumor virology field. Currently, acknowledged human cancer viruses include Epstein-Barr virus, Hepatitis B virus, Hepatitis C virus, High-risk human papilloma viruses, Human T-cell lymphotropic virus type 1 and Kaposi's sarcoma-associated herpesvirus. Extensive epidemiological and mechanistic studies have led to the development of novel preventive and therapeutic approaches for managing some of these infections and associated cancers. In addition, recent advances in molecular technologies have enabled the discovery of a new potential human tumor virus, Merkel cell polyomavirus, but its association with cancer remains to be validated. It is anticipated that in the next few decades many additional human cancer viruses will be discovered and the mechanisms underlying viral oncogenesis delineated. Thus, it can be expected that better tools for preventing and treating virus-associated cancer will be available in the near future.

Keywords

Virus discovery; Cancer viruses; Causality; Epstein Barr virus; EBV; Hepatitis B virus; HBV; Human papillomavirus; HPV; Human T-cell lymphotropic virus type 1; HTLV-1; Hepatitis C virus; HCV; Kaposi's sarcoma-associated herpesvirus; KSHV; Merkel cell polyomavirus (MCPyV)

1. Introduction

At the end of the 19th century, viruses were classified as small infectious agents that, unlike cells and bacteria, pass through fine-pore filters. Subsequently, in 1911 Peyton Rous evidenced an association between cancer and viruses by demonstrating that filtered cell-free tumor extracts could be used to propagate tumors in chickens. Shortly after, however, study of the Rous sarcoma virus (RSV) was suspended and interest shifted to chemical and

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physical initiators of cancer. In 1933, Richard Shope and E. Weston Hurst reported a virus-causing wart on the skin of wild cottontail rabbits. The tumorigenic nature of the disease, now understood to be induced by cottontail rabbit papillomavirus, was subsequently confirmed in collaboration with Peyton Rous. To date, over 100 types of human papillomavirus (HPV) have been identified, several etiologically linked to human cancer. The significant role of viruses in cancer was acknowledged finally in the second half of the past century after various rodent tumorigenic viruses were discovered, and evidence had accumulated supporting an association between viruses and human cancer. Indeed, the Nobel Prize was awarded to Rous in 1966 in recognition of his seminal discovery of tumor-inducing viruses. In addition, almost at the same time, a Special Virus Cancer Program (VCP) was launched by the U.S. congress in 1964 providing enormous funds for intensive research into the supposed role of viruses in human cancer. This program, criticized by some investigators as being a political moonshot-style plan, failed to identify candidate human cancer-causing viruses, yet generated fundamental information about the molecular biology and mechanisms underlying, in particular, virus-related animal cancer, and cancer in general [1].

Currently, six human viruses have been classified by the International Agency for Research on Cancer (IARC) as “carcinogenic to humans” (Group 1) based on sufficient evidence supporting their etiologic association with human cancer: Epstein Barr virus (EBV), hepatitis B virus (HBV), HPV of several types, human T-cell lymphotropic virus type 1 (HTLV-1), hepatitis C virus (HCV), and Kaposi’s sarcoma-associated herpesvirus (KSHV), also known as human herpesvirus 8 (HHV-8) (Table 1, adapted from [2]). The human immunodeficiency virus type-1 (HIV-1) is also listed as a group 1 cancer-causing agent. However, HIV-1 increases primarily the risk of certain malignancies associated with KSHV, EBV and HPV by causing immune suppression. Recent advances in molecular technologies have led to the discovery of a new putative human cancer virus, Merkel cell polyomavirus (MCPyV) [3], though further evidence is required to establish its carcinogenicity. Overall, 12% of the global cancer burden is conservatively estimated to be virus-attributable, with an even higher proportion in developing countries [4]. Since human cancer viruses are often ubiquitous and yet produce tumors in infected individuals only rarely, accumulating enough evidence to establish causal association between any given virus and cancer is a challenge. Therefore, it is likely that the burden of virus-related cancers is underestimated and future research will reveal new associations between already known viruses and human cancer and/or new cancer viruses [5].

Here we review the approaches taken to discover human cancer viruses and promising methods for detecting new viruses. We also discuss how causal association is established and possible cofactors that influence development of virus-associated cancers.

2. Discovery of human cancer viruses

Most of the known human cancer viruses were discovered thanks to unique clinical and epidemiologic clues that pointed to the involvement of an infectious agent in the development of the cancer. Various approaches, ranging from classical virological methods to the most advanced molecular techniques, were used to establish the association between cancer and a given virus. The background and methods underlying the discovery of each human cancer virus are reviewed in this section. Present and future approaches for discovering unknown cancer viruses are presented in section 3.

2.1. Epstein Barr virus (EBV)

EBV was the first human virus to be classified as carcinogenic, a definition based on years of clinical, epidemiological and cell biology studies. Initially, Denis Burkitt reported in the

1950s the appearance of a novel form of childhood B-cell lymphoma in the African malarial belt and hypothesized that the etiologic agent of this disease could be a virus transmitted by an arthropod vector [6]. Then in 1965, Tony Epstein and Yvonne Barr established cell lines from Burkitt's lymphomas and demonstrated by electron microscopy the presence of herpesvirus-like particles in a small fraction of the cells. Werner and Gertrude Henle proved that this virus was biologically and antigenically distinct from other known viruses. However, the high seroprevalence of EBV in human populations worldwide dissuaded researchers from concluding that the virus could be a pathogenic agent underlying African Burkitt's lymphoma. Nevertheless, the initial causal link between EBV and Burkitt's lymphoma was corroborated by the elevated levels of antibodies to EBV antigens present in Burkitt's lymphoma patients. Subsequent studies showed that Burkitt's lymphoma is characterized by a specific chromosomal translocation, t(8;14)(q24;q32) whereby the *Myc* gene is translocated to the active immunoglobulin locus. Ultimately, it became clear that EBV infection provides a key survival signal that sustains outgrowth and clonal expansion at the early stage of the tumor development [7].

Currently, EBV is also associated with nasopharyngeal carcinoma (NPC), Hodgkin's lymphoma, immune-suppression-related non-Hodgkin lymphoma and extranodal NK/T-cell lymphomas. In addition, there is limited evidence supporting an association of EBV with a subset of gastric carcinomas, lymphoepithelioma-like carcinoma and leiomyosarcomas [8–11]. Distinct latency programs characterized by expression of a specific set of viral latent genes are associated with each cancer [12].

2.2. Hepatitis B virus (HBV)

By the beginning of the 20th century it was acknowledged that a transmissible agent capable of causing hepatitis is present in certain blood and blood products. This premise was supported by outbreaks of hepatitis in the 1930–1940s among individuals that had received vaccines containing human sera or plasma. The discovery of HBV itself was serendipitous and not the result of a directed search for the hepatitis-associated bloodborne agent [13]. In 1965, Baruch Blumberg screened sera from multiply-transfused hemophilia patients, who were assumed to have antibodies against polymorphic proteins, for genetic differences in human populations. During the screening, it was found that serum from an American hemophilia patient reacted specifically with an antigen in a blood sample from an Australian aborigine [14]. An association between this Australian antigen, later identified as the HBV surface protein, and the agent causing hepatitis was suspected when a member of the Blumberg's group working with human sera unfortunately contracted hepatitis and went on to manifest seroconversion. Subsequently, in 1967 and 1968, Baruch Blumberg and his colleagues reported that blood samples from patients with hepatitis contained the Australian antigen. In 1976, Blumberg was awarded the Nobel Prize for his discovery of the association of Australian antigen with hepatitis. HBV infection is chronic in a large proportion of individuals. Persistent HBV infection combined with chronic inflammation sometimes results in chronic liver diseases, progression to cirrhosis and eventual development of hepatocellular carcinoma (HCC) [15,16]. Indeed patients with chronic HBV infection are 100-fold more likely to develop HCC than uninfected individuals [17]. The critical proof that HBV can cause HCC was provided by the decline in HCC incidence worldwide following introduction of the anti-HBV vaccine (first licensed in 1982) [18,19].

2.3. Human papillomavirus (HPV)

An association between human warts and viruses was documented as early as 1907 by the Italian physician Giuseppe Ciuffu, who reported that a cell-free filtrate derived from common wart could transfer disease [20,21]. Then in 1935 a link between papillomavirus infection and cancer was demonstrated when Rous and Beard showed that the cottontail

rabbit papillomavirus caused skin carcinomas in rabbits [22]. Further progress concerning this subject was limited by the fairly benign nature of human warts and the lack of a cell culture system to isolate and propagate these viruses. Additional support for the carcinogenic nature of papillomavirus was provided in 1959 when bovine papillomavirus was shown to induce focal transformation of rodent cell lines [23,24], and in 1972 when warts were shown to manifest on skin inoculated with cell-free extracts of skin papillomatous plaques taken from patients presenting with the rare inherited syndrome epidermodysplasia verruciformis (EV). It was already assumed that EV was involved with an infectious agent as unlike most genetic disorders, the geography of family members influences whether they develop symptoms [25], and electron microscopy data had revealed the presence of virus particles in EV plaques [24]. Importantly, it was also known that papillomatous plaques from patients with EV may progress to malignant skin carcinomas and therefore, taking together the EV data validated the suspected link between HPV infections and human cancers.

With regards to human genital papilloma infections, by 1949 it had been demonstrated that genital warts are infectious and contain viral particles. Based on the observation that genital warts infrequently become malignancies, in 1974 Harald zur Hausen proposed that HPV could represent the etiologic agent for cervical cancer in women. In accord with this premise, researchers failed to demonstrate the presence of herpes simplex type 2 DNA in cervical cancer biopsies. Then in the early 1980's, Harald zur Hausen demonstrated by Southern blot analysis the presence of novel HPV types, HPV16 and HPV18, in cervical cancer biopsies as well as in several cervical cancer-derived cell lines, including HeLa cells, the first immortalized human cells grown in culture [26]. Still, it took nearly a decade to prove the causal role of certain HPV types, termed high-risk HPVs, in cervical cancer. Of note, these high-risk HPVs have since been linked to anogenital cancer and to a subset of head and neck cancers. Remarkably, HPV is now recognized as the human cancer virus responsible for causing virtually all cases of cervical cancer in women, with the high-risk HPVs 16, 18, 31 and 45 accounting for up to ~80% of cervical cancer. HPV types 33, 35, 39, 51 52, 56 58 and 59 are also associated with cervical cancer and several other types have been classified as "probably carcinogenic to humans" or "possibly carcinogenic" [2]. HPV vaccines directed against some high-risk strains, including HPV16 and HPV18, are now available, and are expected to limit the spread of HPV infections and the incidence of HPV-related cancers over time. The Nobel Prize in Medicine was awarded in 2008 to Harald zur Hausen for his discovery that human papilloma viruses cause cervical cancer.

2.4. Human T-cell lymphotropic virus type 1 (HTLV-1)

HTLV-1 was the first human retrovirus to be identified and the first to be associated with human malignancy. A distinct type of T-cell leukemia, termed adults T-cell leukemia (ATL), was described in 1977 by Kiyoshi Takatsuki and colleagues in Japanese adults [27]. As with Burkitt's lymphoma, ATL patients cluster in distinct geographic areas suggesting an infectious etiologic agent. In 1980, following an intensive search for human retroviruses, Robert Gallo and colleagues detected reverse transcriptase activity and documented retroviral particles in cultured human T-cell lymphoma cells. The agent proved to be a previously unknown virus, was named HTLV-1 [28]. The association between HTLV-1 and ATL was corroborated when Yorio Hinuma and colleagues demonstrated the presence of extracellular type C viral particles in cells from a patient with ATL [29,30]. Moreover, serologic studies by these investigators and others showed that patients with ATL, but not control individuals, have specific antibodies to antigens expressed in HTLV-1-infected human T cells. Importantly, the geographic distribution of ATL in Japan was found to match the prevalence of HTLV-1 infection [29]. Subsequent studies have confirmed the link between HTLV-1 and cancer [31,32]. HTLV-1 proviral DNA is detectable in virtually every

ATL patient tested. In addition, HTLV-1 infection of normal human T-cells induces cellular immortalization. Finally, the closely related animal retroviruses, bovine leukemia virus and simian T-cell leukemia virus 1, cause leukemia in their respective animal hosts.

Unlike many tumorigenic animal retroviruses that cause cellular transformation by encoding potent oncogenes, HTLV-1 does not encode a classical oncogene. Instead it encodes an essential protein Tax that activates the NF-κB, AP-1 and CRE pathways [31–34]. HTLV-1-related tumors develop decades after primary infection. Of note, following the discovery of HTLV-1, HTLV-2 was isolated from a patient with hairy cell leukemia in 1981, but HTLV-2 has low pathogenicity and is not classified by IARC as carcinogenic. More recently, HTLV-3 and -4 were isolated though the pathogenicity of these viruses remains unclear [35].

2.5. Hepatitis C virus (HCV)

Evidence for a distinct etiologic agent associated particularly with posttransfusion hepatitis began accumulating after the identification of Hepatitis A and B viruses. Indeed, data concerning epidemiology, symptoms and even the viral agent, initially termed ‘non-A, non-B hepatitis’, were available before its actual discovery. A Chimpanzee model that develops acute and chronic hepatocellular damage after inoculation with blood products from affected patients enabled characterization of the ‘non-A, non-B hepatitis’, which was shown to be filterable through 80 nm filters and be susceptible to lipid solvents [36–38]. However, intensive efforts to isolate and propagate the virus in various cell culture models were unsuccessful; in fact, the first *in vitro* HCV propagation system was not developed until 2005. In 1989, following several years of fruitless screens, Michael Houghton and colleagues screened a cDNA phage expression library comprising RNA extracted from the plasma of chronically infected chimpanzees with serum taken from a chronic non-A non-B hepatitis patient. One phage clone was isolated and exhibited a serological profile consistent with the premise that it encoded an antigen component of the non-A, non-B hepatitis. This phage enabled subsequent isolation of overlapping clones from the same cDNA library and led to identification of a new single-stranded RNA virus with a genome of about 10 kb that was called hepatitis C virus [39]. Given the association of HBV with chronic hepatitis and liver cancer, and the similar pathogenesis of ‘non-A, non-B hepatitis’, an association between HCV infection and HCC was soon established.

2.6. Kaposi’s sarcoma-associated herpesvirus (KSHV)

Kaposi’s sarcoma (KS) is a complex multifocal neoplasm, characterized by proliferation of spindle-like endothelial cells, substantial angiogenesis and inflammation. Patients presenting with KS cluster in geographically distinct areas, with high occurrence in certain African countries, moderate in Mediterranean and Eastern European countries, and low in North American and Western European countries. This geographic distribution indicated involvement of an infectious agent in KS and triggered a search for its identity. In the 1980s, frequent occurrence of an aggressive KS form was noted among HIV-infected individuals manifesting acquired immunodeficiency syndrome (AIDS), yet epidemiological and experimental studies did not support a causal association between HIV and KS. Subsequently, an increased incidence rate of KS was noticed specifically among homosexual HIV-infected individuals suggesting that a sexually transmitted agent distinct from HIV might cause KS [40–42]. As with the search for HCV, it was advanced molecular techniques that enabled Yuan Chang, Patrick Moore and colleagues to identify in 1993 the infectious agent involved in KS [43]. A method first described by Lisitsyn and colleagues [44] termed representational difference analysis (RDA) enabled identification of the Kaposi’s sarcoma-associated herpesvirus (KSHV). RDA uses PCR and subtractive hybridization to reveal genetic differences between pairs of biological samples, in particular

between diseased tissue and adjacent unaffected tissue from the same patient. Selective enrichment of DNA sequences unique to KS tissues led to identification of two DNA fragments with homology to sequences of known human herpesviruses and ultimately to characterization of the genomic viral sequence. KSHV was shown to be present in all epidemiologic types of KS, including AIDS-related and AIDS-unrelated KS. Shortly after its discovery, KSHV was shown to be associated with a rare subgroup of B-cell non-Hodgkin lymphoma termed primary effusion lymphoma (PEL), and a subset of multicentric Castleman's disease [45,46]. Subsequently, some KSHV-infected PEL cells were successfully propagated as cell lines facilitating molecular characterization of the virus and development of serologic assays [47,48]. Importantly, diverse prevalence of KSHV infection, which correlates generally with incidence rates of KS, was found in different geographic areas and populations [48–51]. Furthermore, among AIDS patients, KSHV infection was shown to precede KS onset, corroborating a causative role of KSHV in KS [48,52,53]. Finally, in line with the epidemiological data, evidence supporting sexual transmission of KSHV, in particular among homosexual men, was obtained. Of note, the concomitant occurrence of more than one KSHV-related disease in a single patient has been reported providing further validation of the tumorigenic nature KSHV [54,55].

2.7. Merkel cell polyomavirus (MCPyV)

Merkel cell carcinoma (MCC) was the first human cancer found to harbor an integrated polyomavirus genome. MCC, a rare neuroendocrine skin cancer, was observed to occur primarily in elderly and immunosuppressed patients with a relative risk of 13.4 among AIDS patients compared to the general population [56]. In light of the link between immunodeficiency and viral cancers, Yuan Chang and Patrick Moore, the discoverer of KSHV 14 years earlier, sought to identify an infectious agent involved in MCC. The hunt involved the development of a digital transcriptome subtraction (DTS) technique, whereby all transcripts of human origin are subtracted from a set of high-fidelity tumor-derived sequences, revealing the presence of any non-human sequences [3]. Thus, cDNA libraries from MCC tumors were constructed and massively sequenced by high throughput pyrosequencing. Subsequent *in silico* sequence alignment identified a fusion transcript that corresponded to a polyomavirus T-antigen-like sequence contiguous with part of the coding sequence of a human receptor tyrosine phosphatase gene [3]. This transcript was extended using rapid amplification of cDNA ends (RACE) and used to identify the complete sequence of the viral genome. After MCPyV was cloned, several studies corroborated a causal role for this virus in MCC. An extensive survey of MCC biopsies performed by several groups detected the presence of MCPyV in approximately 80% (range 43–100%) of specimens and infrequently in healthy tissues or tissues exhibiting other disease symptoms. Although serologic studies indicated that infection with MCPyV is widespread, MCC patients were found to possess 59-fold higher antibody titers than serologically positive individuals without clinical symptoms [57]. The fusion transcript indicated that the viral genome was integrated in host tumor cell DNA. Further analyses suggested that viral integration occurred prior to clonal expansion and tumor formation. In addition, MCPyV was found to integrate at distinct sites and such genome integration could not be detected in control tissues [58]. Clonal integration of MCPyV in MCC and its metastasis favor the idea of causal relation, which is further supported by the similarity to HPV, in terms of integration into host genome. Of note, other polyomaviruses have been shown to exhibit clonal integration and induce malignant transformation in cultured cells and animal models, however their involvement in human cancers remains controversial [59].

3. Methods for discovering novel cancer viruses

Novel viruses that infect humans are being discovered at a rate of over two per year. Mathematical modeling suggests that we have not yet entered the exponential phase of virus

discovery [60]. Some of the, as yet unidentified, viruses could be human cancer-associated viruses. Besides the classical virology methods based on virus culturing and various molecular methods described above, other approaches involving molecular tools have been employed lately to detect novel viruses. Notably, recent detection methods are not only more sensitive but also allow more than one virus to be identified simultaneously. In general, current molecular methods can be classified into two categories. The first class encompasses methods based on nucleic acid screens for similarities to known viruses and therefore is not expected to identify highly divergent viruses. For example, degenerate PCR using primers that anneal to conserved regions of virus genomes of specific families [61] and panviral DNA microarrays (virochip) [62] are among the methods in this first class. The second category comprises methods not based on nucleic acid homology and hence has the potential to discover highly divergent viruses [63]. A recently developed protocol that falls into this second class aims to purify viral particles through a combination of size selection, density-dependent centrifugation and nuclease treatment before shotgun sequencing (viral metagenomics). This approach has been used to determine viral plethora present in human feces, gut, respiratory tract aspirates and blood [64–67]. An alternative protocol that skips the purification step relies on digital transcriptome subtraction or whole genome analysis to delineate viral sequences after high-throughput sequencing of nucleic acids. Finally, another promising method is based on *in vitro* transposition of circular viral genomes, which enables their propagation in bacteria or cell culture and ultimately, direct sequencing of the genome [68]. These global approaches have the ability to detect low level infections and chromosomally integrated relatively inactive viruses and could reveal complex interactions between pathogens.

The emerging list of cancer viruses is likely to include some zoonoses (virus that infect animals as well as humans) and known viruses that are presently only suspected carcinogens. For example, the human BK and JC polyomaviruses are ubiquitous and widely distributed geographically among the human population. *In vitro* and animal model studies support that these viruses could be associated with different types of human cancers and there are reports that BK and JC are present in certain tumors. Nevertheless, their roles in human cancer remain under debate [59,69]. Similarly, an association between SV40 and human cancer, in particular mesothelioma, has been suggested by several studies that employed PCR-based techniques to detect SV40-specific sequences. Yet, as for BK and JC, this association remains controversial [70]. Future studies that examine not just the presence of viral genomic sequences or proteins but also the genomic integration could help to resolve the role of polyomaviruses in human cancer.

4. Establishing causality

Viral infections, including some by oncogenic viruses, are common among humans. Yet, not all infections by oncogenic viruses lead to cancer. Indeed, the worldwide prevalence of cancer-associated viruses is far greater than the incidence of corresponding neoplastic disorders, while some carcinogenic viruses are ubiquitous in the human populations and others are associated with life-term persistent infection. Furthermore, onset of cancer typically occurs decades after initial infection with a potentially transforming virus. These observations indicate the involvement of other factors in the neoplastic transformation process and that infection is necessary but not sufficient to cause cancer. In other words, cancer is a rare secondary consequence rather than an inevitable outcome of viral infection. This premise is consistent with the general principle that cancer is caused by accumulated genetic and epigenetic changes. Thus, the majority of human cancer viruses appear to function as factors that initiate or promote the oncogenic process as opposed to absolute oncogenes.

The subset of carcinogenic viruses is small compared to the whole set of human viruses. Each cancer-associated virus is etiologically linked to a unique set of diseases presenting with particular characteristics. This supports the idea that unique interactions with cellular targets exist for each cancer virus, though cancer viruses might share common mechanisms for disrupting proper cellular control. Viruses can cause cancer either by inducing or allowing the accumulation of genetic mutations, or by expressing oncoproteins that modify cell survival and proliferation control. A notable exception is KSHV that does not appear to fit into the classical oncogenesis model under immunosuppressive condition. KSHV seems to possess a full spectrum of genetic elements capable of driving infected cells into unlimited proliferation and neoplasia in a very short period without inducing the accumulation of specific genetic alterations [71,72]. Yet, although neoplastic, KS often induces primarily hyperplasia and immortalized cultures are difficult to establish from KS lesions [73]. In most virus-related cancers, immunosuppression such as that associated with HIV infection is a major determinant that predisposes affected individuals to cancers. Yet, no evidence has indicated that HIV actually causes direct cellular transformation. Thus, its role is likely hinged on its ability to regulate the host immune system and microenvironment [74].

Regardless of the mechanism of neoplastic transformation, there is a great challenge to reach an agreement on the etiology for a given human cancer [75]. The challenge is even greater if the given virus is associated with only a subset of a certain type of cancer. For example, HTLV-1 infection commonly occurs at an early age but less than 5% of the infected individuals develop ATL after a long latent period of 20–30 years. It is thought that expression of the TAX protein encoded by HTLV-1 allows accumulation of cellular alterations that promote leukemia development in a small percentage of HTLV-1-infected individuals [31,32,76]. Similarly, only 20% of HCV infected individuals develop cirrhosis and the annual risk to develop HCC is 1–4%, though patients from Japan have a higher risk of developing HCC. It is suspected that chronic inflammation and cirrhosis play a role in promoting HCV-associated HCC [77–79]. Finally, EBV infection is ubiquitous worldwide, however, it is associated with various human malignancies with distinct geographical distributions and involvement of different cell types. In all cases, EBV infection does precede disease onset, and so a variety of environmental and genetic factors have been proposed to contribute to development of each EBV related disease [10].

Given the difference in prevalence versus cancer incidence, mere detection of a virus or its sequence in a particular tumor is not sufficient to unambiguously prove the virus as a causal factor in genesis of the disease. For it remains possible that the virus is ubiquitous among humans and perhaps is drawn to the tumor by the exclusive milieu of cytokines and growth factors therein. Accordingly, guidelines for the evidence required to establish causality have been created. The first formal criteria for microbial disease causation were formulated by Robert Koch and Friedrich Loeffler in 1884 [80]. However, fulfillment of Koch's four postulates has proven difficult for any of the oncogenic viruses discovered to date. Broader epidemiological criteria for causality, proposed by Austin Bradford Hill [81], have become accepted and provide helpful guidelines when assessing potential virus-cancer associations. These criteria are based on: the strength and consistency of association, analogy, specificity, temporality, biological plausibility, coherence with known factors, and experimental verification. In view of the multi-factorial nature of cancer development and host-virus interactions, not all criteria need to be satisfied. Table 2 shows the application of Hill's criteria for establishing an association between KSHV and KS. Importantly, the compelling evidence linking KSHV to KS was generated independently by different research groups. A broad agreement that KSHV is necessary, but not sufficient, to cause KS has been obtained based on Hill's criteria [82].

In light of the demanding criteria for establishing causal association, the oncogenicity of some viruses remains controversial. In the same time, more supporting evidences are needed, that are often ongoing and sometimes complex processes, to allow the attribution of newly discovered pathogens with certain cancer development. For example, there is an ongoing debate concerning the association of Xenotropic murine leukemia virus-related virus (XMRV) with prostate cancer. XMRV was discovered by Anatoly Urisman and colleagues when cDNAs prepared from polyadenylated prostate cancer RNAs were hybridized to a DNA microarray (Virochip) comprising of conserved oligomers of ~950 viral genomes [83]. Seven out of eleven cDNA samples from prostate tumors bearing *RNASEL* homozygous mutated alleles (R462Q) were found positive for the gammaretroviral sequences whereas only one out of 66 tumors from wild-type or heterozygous patients was positive. Following recovery of the entire viral genome and sequence analysis, the novel virus was classified as a xenotropic gammaretrovirus, a designation supported by subsequent ultrastructural studies [84]. Later studies have shown efficient transcriptional activity and replication of XMRV in prostate cancer cell lines as well as a glucocorticoid response element in the U3 region of the XMRV LTR, the latter supporting its tropism restriction to cells expressing the androgen receptor [84,85]. More recently, XMRV infection has been detected in 6% and 23% of prostate cancers by PCR and immunohistochemistry respectively, with higher detection rates in higher grade prostate cancers. However, XMRV infection was shown lately to occur independently of *RNASEL* gene polymorphism, indicating that the general population should be at risk of XMRV infection and its subsequent potential outcome [84]. Future broad epidemiological studies focusing on the prevalence of the virus in malignant tissues and on the natural history of XMRV infection are required to establish a causal relationship between XMRV infection and prostate cancer.

5. Cofactors that influence the outcome of viral infection

Since cancer can be regarded as the exceptional outcome of viral infections, an ongoing challenge is to delineate the concomitant parameters that influence outcome, such as the conditions under which the infection is acquired or the fitness of the infected host. Accordingly, in those cancers that have a viral etiology, infection is regarded as necessary for the development of cancer albeit not sufficient. Often barriers have to be broken before a host succumbs to cancer. At the cellular level, the status of cell cycle checkpoints and apoptosis, senescence and autophagy pathways affect the outcome of infection. At the organism level the host's immune system comprising innate, humoral and cellular pathways, are perhaps the most important guard against cancer development, especially in the context of virus-related cancers [74]. Accordingly, increased incidence of virus-related cancers is evident in elderly and immunosuppressed individuals, with the incidence exceptionally high among AIDS patients. The latter finding could be the result of HIV induced immunosuppression only or could also be due to mutual molecular interactions between HIV and other pathogens. Indeed, AIDS-related KS, PEL and MCD cancers have been shown to occur disproportionately among untreated HIV-positive/KSHV-infected individuals. It is suspected that HIV-1 and KSHV influence one another and disease progression through co-dependent activation of a KSHV immediate-early gene promoter and the HIV-1 LTR [86–88]. Co-infection with other pathogens, particularly those that cause chronic inflammation, likely play a crucial role in certain virus-related cancers.

Endemic patterns of incidence of several virus-related cancers may reflect differences in the prevalence of the causing virus. Yet, the geographically limited distribution of some viral cancers despite the prevalence of viral infection, together with the rare cases of familial virus-related cancers, suggests genetic and environmental factors influence the outcome of viral infections. Indeed, a role for host genetic and environmental factors is corroborated by the converse observation that certain populations demonstrate resistance to the

tumorigenesis induced by some viruses. In particular, it is speculated that Human Leukocyte Antigen (HLA)-dependent immune recognition is involved in controlling virus-infected cells and that viral strains adapt to it by expressing different HLA epitopes. Accordingly, HLA type likely affects the outcome of infection [89–91]. Future identification of host genes and pathways that confer susceptibility or resistance to a certain virus could not only uncover mechanisms of oncogenesis but also facilitate development of effective diagnostic and therapeutic tools.

For example, various studies have begun to unravel the cofactors influencing the outcome of EBV infection. The plethora of host genetic and environmental cofactors that influence the incidence of EBV-related cancers illustrates the complex pathogenesis of viral cancer. One important cofactor in the development of Burkitt's lymphoma is co-infection with malaria parasites, which induce expansion of germinal center lymphocytes, which in turn expands the cellular pool susceptible to EBV infection [92]. With regards to EBV-associated NPC, this cancer has been correlated with increased antibody titers to selected EBV antigens along with increased levels of circulating free-virus. The highest incidence of NPC is among Cantonese people in Southern China and natives in the Arctic region, exemplifying the significance of genetic or environmental cofactors in virus-induced cancers. Indeed, NPC-promoting factors have been identified in food products, such as salt-preserved fish, which are commonly consumed in Southern China and in herbal medications taken by the affected Chinese populations. Other risk factors such as tobacco, other smokes, alcohol consumption and occupational exposures have also been implicated as influencing the outcome of EBV infection. Finally, familial occurrence of NPC has been documented supporting that genetic and/or shared susceptibility factors affect the outcome of EBV infection. Indeed, detailed segregation analysis of familial NPC in Southern China evidenced a multifactorial genetic and environmental background underlying this clustering [93].

6. Perspective

The last four decades have witnessed rapid advancement of the tumor virology field, from discovery of human oncogenic viruses to demonstration of causality and evaluation of mechanisms. As a consequence, novel preventive and therapeutic approaches have been developed for some cancer-associated oncogenic viruses. The paradigm was the HBV vaccine, first produced in 1980, that represents the first vaccine capable of preventing a specific human cancer. Similarly, the recent introduction of HPV vaccines is expected to prevent cervical cancer, the third leading cause of cancer-related mortalities in women worldwide, and perhaps will prevent other HPV-related cancers. This remarkable progress should translate into overall reductions in cancer virus infections, in the incidence of viral cancers and in morbidity and mortality of relevant cancer populations, nevertheless much remains to be done. Development and implementation of novel preventive procedures based on the known biology and epidemiology of other cancer viruses is a high priority. Many research groups are focused on the search for therapeutic targets and novel vaccines and these efforts must continue and expand. In parallel, several unresolved controversies concerning causality should be clarified such as the association between XMRV and prostate cancer, EBV and breast cancer, and polyomaviruses and various cancers. It can be envisaged that new viruses will continue to be discovered over the next several decades as more sophisticated molecular technologies are developed. Of particular note, up to 30% of the human genome contains sequences from endogenous retroviruses and it remains to be determined whether reactivation of some of these and/or expression of constituent genes is associated with certain cancers. Immunosuppressed individuals, such as AIDS patients, offer a unique opportunity to uncover such associations and discover new viruses. However it must be remembered that the discovery of new viruses represents just the beginning of a long research journey as the task of proving causality remains enormous due to the many

cofactors that influence the outcome of infection. The next decade promises to be an exciting era for the tumor virology field.

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Table 1

Human cancer viruses.

Virus	Year of Discovery	Disease associated with primary infection	Acknowledged associated human cancers	Suspected associated human cancers
Epstein Barr virus (EBV, human herpesvirus 4 [HHV-4])	1965	Asymptomatic infection, Infectious mononucleosis	Burkitt's lymphoma, Nasopharyngeal carcinoma, Hodgkin's lymphoma, immunosuppression-related non-Hodgkin lymphoma, extranodal NK/T-cell lymphoma	Gastric carcinoma, lymphoepithelioma-like carcinoma, leiomyosarcomas
Hepatitis B virus (HBV)	1967–8	Asymptomatic, acute hepatitis, long-term chronic infection of the liver	Hepatocellular carcinoma	Cholangiocarcinoma, non-Hodgkin lymphoma
Human T-cell lymphotropic virus type 1(HTLV-1),	1980		Adult T-cell leukemia and lymphoma	
Human papillomavirus (HPV) (high-risk types)	1983		Carcinoma of the cervix, vulva, vagina, penis, anus, oral cavity, and oropharynx and tonsil	Cancer of the larynx, and some head and neck cancers
Hepatitis C virus (HCV)	1989	Asymptomatic, acute hepatitis	Hepatocellular carcinoma, non-Hodgkin lymphoma	Cholangiocarcinoma
Kaposi's sarcoma-associated herpesvirus (KSHV, human herpesvirus 8 [HHV-8])	1994		Kaposi's sarcoma, primary effusion lymphoma	multicentric Castleman's disease
*Merkel cell polyomavirus (MCPyV)	2008			Merkel cell carcinoma

*Further evidence required for unquestionable inclusion as an etiologic agent of cancer.

Table 2

Criteria and evidence used to weigh the association between KSHV and KS.

Criteria	Evidence
Strength of the association (association between an agent and disease)	Almost all KS lesions, from different settings, contain the KSHV genome, whereas it is rarely found in control and adjacent tissues (>100 odds ratio for detecting KSHV DNA in KS lesions).
Consistency of disease association (consistent findings across studies)	Detection of KSHV genome, transcripts and proteins in KS lesions by hundreds of independent investigators. KSHV is present in all clinical and epidemiologic types of KS. Worldwide, the prevalence of KSHV is correlated with KS incidence.
Analogy	KSHV is closely related to viruses with established tumorigenic potential, including EBV and simian rhadinoviruses. The latter have been associated with retroperitoneal fibromatosis, which pathologically resembles KS.
Specificity of the association (infection associated with selected cancers)	KSHV is uncommon in populations presenting low occurrence of KS and vice versa. KSHV is also associated with primary effusion lymphoma and multicentric Castleman's disease, but not with common diseases. Several reports on concomitant occurrence of these diseases in the same patient have been reported.
Temporality (infection precedes disease onset)	Infection with KSHV precedes KS onset. KSHV viremia and increased antibody titers to lytic antigens (an indication of active lytic replication) predict KS development.
Biological plausibility (does the association seem reasonable when referring to the current knowledge)	KSHV encodes several potential transforming genes, some of which share homology with known cellular regulators of cell fate. Viral proteins are expressed in KS lesions.
Coherence	A strong correlation between prevalence of KSHV infection and the overall pattern of occurrence of KS. High rates of disease in immunocompromised patients, particularly AIDS patients.
Experimental evidence	AIDS KS incidence may be reduced with ganciclovir therapy.