Viruses in human cancers*

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Infections have been recently identified as important etiologic factors for an increasing percentage of human cancers. Between 15 and 20% of the global burden of human cancers have been linked to viral, bacterial and parasitic infections. Viruses play a major role, since two types of infections, anogenital papillomavirus types and Hepatitis B virus account for about 15% of cancers in females and slightly less than 10% of cancers in males. This review summarizes the criteria used for the identification of carcinogenic infectious agents, analyzes some of the known mechanistic contributions of viruses to carcinogenesis, presents some host and host cell responses to these infections, and discusses prospects for the prevention and therapy of virus-linked cancers.

Presently between 15 and 20% of the global cancer incidence can be etiologically linked to specific infections. Bacteria (Helicobacter pylori) and helminths (Schistosoma, Opisthorchis, Clonorchis) contribute to the development of bladder and rectal cancers and to cholangiocarcinomas. Viruses, however, are the main cancer risk factors. Hepatitis B and C virus infections are involved in hepatocellular carcinomas. Specific types of papillomaviruses cause one major human cancer, cancer of the cervix and play an additional role in a number of other anogenital, but also in some oropharyngeal and cutaneous cancers. Epstein–Barr virus, known as a human tumor-virus since 1964, human herpes-virus types 8 (HHV-8), and human T-lymphotropic retrovirus type 1 (HTLV-1) have also been identified as human tumor-viruses.

There still remain some tumor types as candidates for a possible infectious etiology: this includes lymphomas and leukemias, but also some epithelial tumors. Human tumor-viruses are commonly ubiquitous. A low rate of infected individuals eventually develops the respective form of cancer. Malignant conversions, usually the consequence of additional genetic modifications in latently infected cells, occur under conditions of severe immunosuppression. It is exceedingly difficult to reveal the existence of as yet unknown tumor-viruses by epidemiological studies if these are ubiquitous. Their discovery will depend on sensitive molecular biological or immunological approaches. In addition, the possible transmission of animal tumor-viruses to humans which may only be able to express transforming genes in human cells, has only been scarcely investigated and cannot be ruled out.

Criteria to link a virus infection to the etiology of a human tumor

The presence of viral DNA within human tumor biopsies may serve as a hint of an etiological relationship. The same is true for seroepidemiological studies revealing elevated antibody titers against a specific virus. Table 1 lists some of the methods for the discovery of the role of human viruses in cancer induction.

Characteristic features in the development of virus-linked tumors add to the difficulties to identify the causative agent. These cancers commonly arise only after long latency periods of several decades between primary infection and development of the respective neoplasm. The tumors are clonal, their monoclonality can frequently be deduced from the integration pattern of viral DNA. Most of the infected individuals either clear the infection by immunological interference or harbor viral DNA for lifetime within specific cells without symptoms.

Viral DNA frequently persists in subgenomic fragments in virus-positive tumors. These tumors are consequently unable to give rise to infectious progeny. Therefore, Koch’s postulates to causally imply a bacterial infection in a specific disease cannot be applied to tumor-viruses. They were based on the isolation of the infectious agent, its in vitro propagation, the re-inoculation into a susceptible animal host and the induction of symptoms analogous to those observed in the diseased patient.

Attempts have been made to account for these difficulties and to define new criteria linking virus infections to human cancer, most frequently involving epidemiological and seroepidemiological data. The different modes of virus contributions to cell transformation, including their

*Table 1. Methods used for the initial discovery of human tumor-viruses and early data relating to their carcinogenic potential

<table>
<thead>
<tr>
<th>Virus Type</th>
<th>Method</th>
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<tbody>
<tr>
<td>Liver cancer and hepatitis B</td>
<td>Epidemiology</td>
</tr>
<tr>
<td>Cervical cancer and HPV</td>
<td>Molecular biology and epidemiology</td>
</tr>
<tr>
<td>Lymphomas and Epstein–Barr virus</td>
<td>Serology, molecular biology, epidemiology</td>
</tr>
<tr>
<td>Adult T-cell leukemia</td>
<td>Molecular biology, serology, epidemiology</td>
</tr>
<tr>
<td>Kaposi sarcoma and HHV-8</td>
<td>Molecular biology</td>
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Vaccinations will aid in case of hepatitis B and papillomavirus infections.

*A modified form of this manuscript has been published before in the European Journal of Cancer.
role as direct or indirect carcinogens render such efforts rather difficult.

The following four criteria\textsuperscript{3,4} can be only considered as valid for those tumor-virus infections that permit the identification of a trans- or cis-acting viral gene or DNA-fragment and exclude all indirect contributions to carcinogenicity, as for instance by continuing immunosuppression (e.g. HIV infections). (i) Epidemiological plausibility and evidence that a virus infection represents a risk factor for the development of a specific tumor; (ii) Regular presence and persistence of the nucleic acid of the respective agent in cells of the specific tumor; (iii) Stimulation of cell proliferation upon transfection of the respective genome or parts thereof in corresponding tissue culture cells; (iv) Demonstration that the induction of proliferation and the malignant phenotype of specific tumor cells depend on functions exerted by the persisting nucleic acid of the respective agent.

Particularly the last point represents the most stringent criterion to link an infection to tumors and to separate it from co-factors.

Mechanisms of virus-mediated cell transformation

Several human tumor-viruses induce continuous proliferation (immortalization) of specific human tissue culture cells. Immortalization uniformly precedes malignant conversion which cannot be achieved in a single step. We define here malignant growth as invasive proliferation after heterotransplantation into immunocompromised animals. During the past few years it became increasingly clear that individual steps resulting in immortalization and eventually in a malignant phenotype include modifications of specific cellular genes, part of them engaged in the control of the persisting viral genome. Here some known functions of viral oncogenes will be discussed. Table 2 lists human tumor-viruses and other human virus infections, the latter are carcinogenic in experimental animal systems without a similar role in humans.

Prospective epidemiological studies underline the role of hepatitis B and C viruses in the etiology of hepatocellular carcinomas. Yet, these viruses are unable to immortalize human cells in tissue culture. In spite of the definition of trans-activating functions of specific HBV and HBC proteins (see below), their contribution to malignant conversion is presently unknown.

Similarly, little is known of viral functions contributing to the malignant conversion of lesions associated with papillomavirus types (HPV 5, 8, 14, 17, 20 and a few others) in epidermodyplasia verruciformis (EV) patients. In contrast to anogenital malignant tumors, carcinomas in EV patients only exceptionally seem to contain integrated viral DNA\textsuperscript{5}. The preservation of $E_6$ and $E_7$ genes under these conditions may suggest a similar important role as demonstrated for high risk anogenital HPV infections. Very recent data suggest that the E6 protein of some of these viruses blocks apoptosis after exposure to DNA damage, e.g. by ultraviolet (UV) light\textsuperscript{6}. In addition, the promoter region is activated by UV exposure\textsuperscript{7}. Since squamous cell carcinomas of the skin most commonly arise on sun-exposed sites, some of these viruses may contribute to malignant growth by an indirect mechanism.

The human polyomavirus types BK and JC, as well as various types of human pathogenic adenoviruses have not been consistently found in human tumors, they are however carcinogenic for newborn rodents. Under specific circumstances they are able to immortalize human cells, usually very inefficiently.

Epstein–Barr virus (EBV), human papillomaviruses (HPV), human T-lymphotropes virus (HTLV)-1, and human herpes-virus (HHV)-8 possess defined oncogenes that stimulate proliferation of specific human cells. Although trans-activating properties have been defined for the HBV X and pre-S antigens\textsuperscript{8-10} and mice transgenic for these genes and those transgenic for the HBC core antigen develop hepatocellular cancers\textsuperscript{11}, it is presently unknown whether and to which extent the same genes contribute to human liver cancers.

Epstein–Barr virus infection of B lymphocytes in vitro requires at least 6 viral genes (EBNAs 1, 2, 3A, 3C, LP, in LMP1) for cell immortalization\textsuperscript{12}. In in vivo infections this expression seems to be characteristic for infectious mononucleosis\textsuperscript{13}. Three modes of EBV latency have been defined\textsuperscript{14}. Specifically in EBV-positive Burkitt’s lymphomas latency program I is expressed, permitting only the synthesis of the Epstein–Barr virus nuclear antigen (EBNA)-1, transcripts clustered around one other open reading frame, BARF-0, and a small abundant non-translated RNA, EBER. EBNA-1 is required for EBV DNA replication and to permit the persistence of episomal viral DNA, the role of transcripts in the BARF-0 region is still not elucidated. Interestingly, recent data seem to point to a specific role of EBER transcripts in preventing apoptosis (Takada, pers. commun). In most other EBV containing malignant tumors (nasopharyngeal cancer, EBV-positive Hodgkin’s lymphomas and gastric cancers) latency program II is expressed, corresponding to the previous one, but expressing in addition to a varying degree the latency membrane proteins (LMP) 1 and 2. Latency program III finally is observed in EBV-induced lymphoblastoid proliferation and in EBV-positive B cell lymphomas arising under conditions of immunosuppression. In this case gene regulation occurs from a different promoter (Cp/Wp) and results in the expression of 6 different EBNAs, three latent membrane proteins and EBER RNA. The EBNA-2 protein represents a specific transactivator of cellular and viral genes. It binds to the cellular protein RBP-Jk which acts downstream of the Notch-signaling pathway and transforms this protein from a repressor into an activator\textsuperscript{5,16}. 

\begin{table}
\centering
\begin{tabular}{|c|c|c|c|c|}
\hline
\textbf{Virus} & \textbf{Oncogene} & \textbf{Function} & \textbf{Target} & \textbf{Expression} \\
\hline
\textsuperscript{5}EBV & $E_6$ & Block apoptosis & DNA damage & \textsuperscript{6}UV light \\
\textsuperscript{7}HHV-8 & Transgenic & Stimulation proliferation & Human cells & Efficiently \\
\textsuperscript{8-10}HBV & Pre-S & Trans-activating & Specific human cells & Efficiently \\
\end{tabular}
\end{table}
The mechanism by which EBV proteins contribute to malignant tumors is still poorly understood. This account in particular for tumors expressing latency programs I or II. EBV-positive lymphomas developing in immunosuppressed patients emerge as the result of a failing immune system and the potent transactivating and growth-stimulating activity of EBNA-2.

HHV-8 is the most recently identified human tumor virus. Its genome contains a number of genes whose products are related to cellular cyclins (cyclin D), to cytokines (II-6) and to interferon-responsive factors (IRF-2). Their accurate contribution to growth-stimulation and cell transformation is presently under intensive study.17-19.

HTLV-I and also a related retrovirus, not yet linked to human tumors, HTLV-II, are both able to immortalize human T lymphocytes.20,21. This property seems to relate to a specific viral gene tax that has been identified as a transforming factor,22, possessing strong transactivating properties.23,24

High risk human papillomavirus, particularly well studied HPV 16 and 18, code for three viral oncoproteins. One of them, E5, is obviously not required to initiate and maintain the malignant phenotype of cervical carcinoma cells. It seems to play, however, a role in early growth stimulation of cells infected by these viruses. The two other oncogenes, E6 and E7, of high risk types are able to immortalize human keratinocytes in contrast to low risk types. They contain all the necessary information for cell immortalisation,25 although infection of susceptible cells or transfection of these genes per se is not sufficient for the induction of an unlimited in vitro life span. The E7 protein interacts with the retinoblastoma susceptibility gene product pRb (ref. 26) and other pRb-related proteins. As a consequence, it interrupts a complex between pRb and E2F, releasing the E2F transcription factor which activates genes engaged in cell cycle progression. Additional binding activities have been described for E7 whose functional importance has not yet been clearly established. The E6 protein binds p53 and abrogates its tumor suppressive and transcriptional activation properties.27 It promotes ubiquitination of p53 and its subsequent proteolysis through interaction with the E6AP ubiquitin–protein ligase.28 E6 and E7 are able to immortalize human keratinocytes independently, although both genes co-operate effectively in immortalization events. As observed for E7, E6 also targets other proteins: the focal adhesion protein paxillin and the interferon regulatory factor 3 (IRF-3), blocking the induction of interferon-βmRNA after viral infection.3 These data indicate the multi-functional nature of viral oncoproteins, modifying a multitude of cellular functions. Table 2 summarizes known human tumor-viruses.

Protective mechanisms of the host against potential tumor-viruses – the CIF-concept

The discussion on the role of EBV in Burkitt’s lymphoma has been controversial for more than 30 years. Recently some direct evidence has been reported pointing to an EB viral contribution to the malignant phenotype of these cells. Loss of EBV genomes was noted in the BL line Akata.32 EBV negative clones lost the tumorigenic phenotype which was reconstituted by reintroduction of EBV DNA.

Evidence was obtained recently for the host regulation of persistent EBV infections by the genetic analysis of a rare EBV-linked condition, the X chromosome-linked lymphoproliferative syndrome (XLP).33 In XLP patients the host is unable to cope with the B cell proliferation which characterizes the initial stage of infectious mononucleosis, a disease caused by acute EBV infection. XLP patients succumb from an enormous lymphoproliferation. The condition affects exclusively young males as has been linked to deletions of the X chromosome. Recently mutations have been identified in an inhibitor of a protein (SLAM) that regulates T/B cell interactions. The gene for the inhibitor protein, SLAM-associated protein or SAP, was identified at the site of the X chromosome deleted in XLP patients. A subsequent analysis demonstrated its mutation specifically in this group of patients. It is presently not understood, how this disturbance in T/B cell relatively selectively affects the T cell control of EBV infections.

Probably the best evidence for host defense mechanisms against a family of tumor-viruses, even beyond a mere immunological control, has been derived recently from studies on human papillomavirus infections. The papillomavirus family reveals an enormous complexity with 85 fully analysed genotypes and more than 120 additional putative genotypes which are only partially characterized up to now (de Villiers, pers. commun). It seems that the peculiar mode of papillomavirus propagation at cutaneous and mucosal surfaces only marginally exposes these viruses to the immune system of the host. This may release these infections from immunological constraints acting on other systemic infections and may in part account for the puzzling multitude of HPV genotypes.

Table 2. Established human tumor-viruses and human viruses causing tumors only under experimental conditions

<table>
<thead>
<tr>
<th>Type of Virus</th>
<th>Remarks</th>
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<tbody>
<tr>
<td>Directly carcinogenic viruses</td>
<td></td>
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<tr>
<td>Epstein-Barr virus</td>
<td></td>
</tr>
<tr>
<td>Human herpes-virus type 8</td>
<td></td>
</tr>
<tr>
<td>Several anogenital papillomavirus types</td>
<td>(e.g. HPV 16, HPV 18 and</td>
</tr>
<tr>
<td>(e.g. HPV 16, HPV 18 and others)</td>
<td>others)</td>
</tr>
<tr>
<td>HTLV-I</td>
<td></td>
</tr>
<tr>
<td>Hepatitis B (?)</td>
<td></td>
</tr>
<tr>
<td>Indirectly carcinogenic viruses</td>
<td></td>
</tr>
<tr>
<td>HIV</td>
<td></td>
</tr>
<tr>
<td>Hepatitis B (?) and hepatitis C</td>
<td></td>
</tr>
<tr>
<td>Some cutaneous papillomavirus types (?)</td>
<td></td>
</tr>
<tr>
<td>Human viruses carcinogenic in experimental</td>
<td></td>
</tr>
<tr>
<td>animals</td>
<td></td>
</tr>
<tr>
<td>Human polyomaviruses BK and JC</td>
<td></td>
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<tr>
<td>Several types of human adenoviruses (e.g.</td>
<td></td>
</tr>
<tr>
<td>adenovirus type 12, type 18 and</td>
<td></td>
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<tr>
<td>others)</td>
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Previous studies showed that E6/E7 gene expression of high risk HPV types is necessary for the initiation and maintenance of the immortalized but also for the malignant phenotype of HPV DNA-carrying cells. These findings, combined with additional observations demonstrated that the expression of viral oncoproteins and transcription of viral oncogenes is necessary but not sufficient for the immortalized and malignant state of HPV-infected cells. Somatic cell hybrids between HPV-immortalized clones or between SV40-immortalized clones revealed the existence of complementation groups complementing each other to cellular senescence in spite of continuing E6/E7 mRNA or SV40 T-antigen synthesis. Therefore a hypothesis was put forward postulating a cellular control of viral oncogene transcription or viral oncoprotein function, preventing in proliferating cells of the natural host their potential transforming and thus deleterious effect for the host. For high risk HPV infections a possible transcriptional control received experimental support from data showing suppression of HPV transcription upon inoculation of immortalized cells into immunocompromised animals and from a barely detectable level of E6/E7 transcripts in most low grade cervical intraepithelial lesions in contrast to high grade dysplasias. The initial suspicion of one cellular interfering factor (CIF) exhibiting an intracellular control beyond an immunological surveillance had to be modified into a CIF-concept.

Today there exists good evidence in support of the CIF-concept: at least two signaling cascades emerge regulating the transcription of high risk HPV oncogenes and interfering with the function of viral oncoprotein. A functional control can be deduced from somatic cell hybridization studies revealing continued HPV mRNA synthesis in senescent hybrids of initially immortalized clones. Transfection of human keratinocytes with the HPV 16 E6 oncogene only results in immortalized cells that generally contain mutated or methylated-silenced sequences of the p16

\[ \text{cyclin-dependent kinase inhibitor} \]

(Whitaker and zur Hausen, unpublished data). This suggests an important role of p16 in the control of E6-mediated cell immortalization. The regulatory steps engaged here are still not understood. For E7 as well as for E6 and E7 immortalized cells the obviously existing functional interference is even less understood. A high level of p21

\[ \text{CIP-1} \]

and p27

\[ \text{CIP-1} \]

may negatively interfere with a low level of E7 expression, at least deregulated E7 expression in turn is able to inactivate these cyclin-dependent kinase inhibitors reciprocally.

The CIF-cascade interfering with the transcription of persisting high risk HPV is somewhat better understood. Exposure of HPV 16- or 18-immortalised cells to macrophages or treatment with tumor necrosis factor (TNF) \[ \alpha \] leads to a selective suppression of HPV transcription. The effect is absent in HPV-containing malignant cervical cancer cells. Suppression in HPV transcription is accompanied by a remarkable shift in the composition of the AP-1 transcription factor as one of the important regulators of the HPV genome activity. In immortalized cells in tissue culture predominantly c-jun/c-jun homodimers form this complex, whereas in most malignant lines c-jun/c-fos heterodimers prevail. TNF\[ \alpha \] treatment results in the induction of a c-fos analogon, Fra-1, which now heterodimerizes with a phosphorylated form of c-jun. Malignant cells neither reveal Fra-1 induction nor an increase in c-jun/Fra-1 heterodimers. Although not directly proven, the available data suggest that the change in the AP-1 composition is responsible for the observed selective inhibition of HPV transcription in immortalized cells.

Presently a tentative CIF-cascade can be deduced from data available in the literature: there exists evidence that the protein phosphates 2A (PP2A) plays an important role in the regulation of HPV transcription. This is derived from data published by Smits et al. who demonstrated that human cells carrying a deletion in the short arm of chromosome 11 revealed a up-regulation of the regulatory component of PP2A, the PR55\[ \beta \] protein. The resulting down-regulation of PP2A function that was also achieved by ocdacic acid treatment or by the introduction of SV40 small t-antigen led to increased transformability of these cells by high risk HPV DNA transfection and high levels of E6/E7 gene transcription. Preliminary data from our laboratory suggest that conditional up-regulation of E7 expression in turn activates the PR55\[ \beta \] gene, pointing to a positive feedback mechanism (Hoffmann and zur Hausen, unpublished data). The activation of MKK6/p38 kinases by TNF\[ \alpha \] (ref. 56) may point to an important role of these kinases in the downstream events resulting in up-regulation of FRA-1 and c-jun.

The data reported here reveal a control of persisting natural tumor-virus infections that are not readily reached by the immune system by intra- and intercellular signaling cascades. In these instances cancer represents an accident resulting from modifications of host cell genes involved in the control of viral oncogenes and oncoproteins. In high risk HPV infections the viral oncoproteins, in addition to their gene regulatory functions appear to act as mutagens and may thus act as solitary carcinogens.

**Perspectives for cancer prevention and cancer therapy**

The discovery of infectious agents as causative factors for specific human cancers has important consequences for cancer prevention. This is presently apparent from vaccination studies performed in Taiwan and The Gambia. The Taiwan vaccination program of newborn children against hepatitis B infections, introduced in 1986, not only drastically reduced the percentage of persistently HBV-infected children, but also resulted in a first measurable decrease in liver cancer incidence. Presently clinical trials determine the efficacy of vaccines directed against high risk papillomavirus infections.
tests in animal papillomavirus infections, based on analogous vaccine preparations revealed a remarkable efficiency. The first tests in human clinical trials revealed the safety of the vaccine and the induction of high titers of neutralizing antibodies. Provided the vaccines against human high risk HPVs prove to be similarly effective as in animal experiments, one can estimate that a global application of HPV and HBV vaccines could theoretically reduce the cancer risk in women by approximately 15%.

Clinical trials are also presently conducted to test the potential of therapeutic vaccines. Their chances for effectiveness may more concern pre-malignant lesions rather than fully invasive tumors, although this remains presently difficult to predict.