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How virus persistence can initiate the tumorigenesis process

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Abstract

Human oncogenic viruses are defined as necessary but not sufficient to initiate cancer. Experimental evidence suggests that the oncogenic potential of a virus is effective in cells that have already accumulated a number of genetic mutations leading to cell cycle deregulation. Current models for viral driven oncogenesis cannot explain why tumor development in carriers of tumorigenic viruses is a very rare event, occurring decades after virus infection. Considering that viruses are mutagenic agents *per se* and human oncogenic viruses additionally establish latent and persistent infections, we attempt here to provide a general mechanism of tumor initiation both for RNA and DNA viruses, suggesting viruses could be both necessary and sufficient in triggering human tumorigenesis initiation. Upon reviewing emerging evidence on the ability of viruses to induce DNA damage while subverting the DNA damage response and inducing epigenetic disturbance in the infected cell, we hypothesize a general, albeit inefficient hit and rest mechanism by which viruses may produce a limited reservoir of cells harboring permanent damage that would be initiated when the vi-

rus first hits the cell, before latency is established. Cells surviving virus generated damage would consequently become more sensitive to further damage mediated by the otherwise insufficient transforming activity of virus products expressed in latency, or upon episodic reactivations (viral persistence). Cells with a combination of genetic and epigenetic damage leading to a cancerous phenotype would emerge very rarely, as the probability of such an occurrence would be dependent on severity and frequency of consecutive hit and rest cycles due to viral reinfections and reactivations.

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Key words: Virus; Carcinogenesis; Tumor; Oncogene; Latency; Viral persistence

Core tip: Current models for viral driven oncogenesis cannot explain why tumor development in carriers of tumorigenic viruses is a very rare event, occurring decades after virus infection. Considering that viruses are mutagenic agents *per se* and human oncogenic viruses additionally establish latent and persistent infections, we attempt here to provide a general mechanism of tumor initiation both for RNA and DNA viruses, suggesting viruses could be both necessary and sufficient in triggering human tumorigenesis initiation.

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TUMORS AND VIRUSES

According to currently accepted estimates, viruses are etiologically linked to 15%-20% of all cancer cases worldwide^[1-3]. Although many animal and human viruses can transform cells upon infection, only six human viruses are consistently associated with the onset of tumors in man,

Table 1 Oncogenic viruses are latent/persistent viruses

Virus	EBV	HHV-8	HPV	HBV	HCV	HTLV-1
Associated tumor(s): viral protein(s) expressed	BL: EBNA-1 NPC, TCL: EBNA1 + LMP1 HL: EBNA1 + LMP1-2 PTLD: EBNA1-6 + LMP1-2	KS: vFLIP, vCYC, LANA-1 PEL, MCD: vFLIP, vCYC, LANA-1, LANA-2, vIL-6	Anogenital, oral, skin and laryngeal cancers: E6, E7	HCC: HBx	HCC: CP10, NS3, NS5	ATL: tax
Persistency	Always	Always	20% infected subjects	90%-95% infected newborns 5% infected adults	70%-85% infected subjects	Always
Period between infection and tumor onset	10-20 yr	10-20 yr	5-20 yr	10-30 yr	10-30 yr	20-30 yr

EBV: Epstein-Barr virus; HHV-8: Human herpesvirus 8, also named Kaposi sarcoma virus; HPV: Human papillomavirus; HBV: Hepatitis B virus; HCV: Hepatitis C virus; HTLV-1: Human T-cell leukemia virus 1; BL: Burkitt lymphoma; NPC: Nasopharyngeal carcinoma; TCL: T cell lymphoma; HL: Hodgkin lymphoma; PTLD: Posttransplant lymphoproliferative disorder; KS: Kaposi sarcoma; PEL: Primary effusion lymphoma; MCD: Multicentric Castleman's disease; HCC: Hepatocellular carcinoma; ATL: Adult T-cell leukemia.

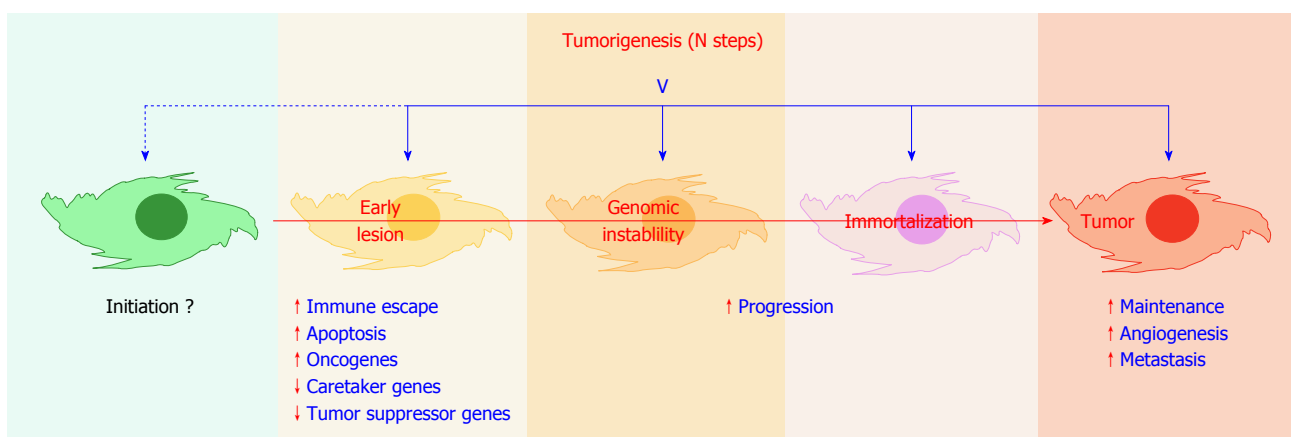


Figure 1 Viral infection and tumorigenesis. Viruses have been shown to encode functions that can modulate all crucial steps towards tumor development, with the exception of the initiation step(s). Recognized contributions of viral infection are mentioned in blue letters. V: Virus. Red arrowheads up, stimulation; down, inhibition.

namely human papillomavirus (HPV), human T-cell lymphotropic virus 1 (HTLV-1), Epstein-Barr virus (EBV), human herpesvirus 8 (HHV-8), hepatitis B virus (HBV) and hepatitis C virus (HCV) (Table 1). A large share of what is known about the molecular mechanisms of oncogenesis is due to studies of tumor viruses, defined thereof as viruses carrying in their genome one copy of an oncogene or of an anti-oncogene or viruses that can alter the expression of the cellular version of one such gene^[4]. Viruses have been shown to influence tumor sustainment and progression and induce escape pathways from apoptosis and immune surveillance^[1,4], however in no case has it been proven that a virus can be the initiator, the primum movens, and not merely an “influential passenger” of a tumor (Figure 1).

TUMORS AND GENES

Tumor development is believed to be a multistep process leading to the accumulation of permanent genetic damage^[5], affecting either oncogenes, tumor suppressor

genes, or stability genes^[6,7]. Cancer is therefore essentially a genetic disease, and a crucial observation in understanding multistep carcinogenesis is that the vast number and the coarse/crude nature of chromosomal defects that are present in the majority of tumor cells^[8], are not amenable to an altered mutation rate in these cells^[9,10]. In fact, most human solid tumors are characterized by an abnormal chromosome content, aneuploidy, which can be caused by genetic instability^[8,11,12]. In addition, distinct and inheritable gene expression and phenotypic states that arise independently from changes in DNA sequence, known as epigenetic modifications, are also linked to tumor formation and progression^[13,14]. On the whole, mechanisms for the initiation of tumorigenesis leading to genetic instability are on the whole poorly understood, both for virus induced and virus unrelated tumors^[6].

CAN VIRUSES INITIATE GENETIC INSTABILITY?

It has been known for more than four decades that

Table 2 Viral proteins inducing genetic damage

Virus	EBV	HHV-8	HPV	HBV	HCV	HTLV-1
Latent proteins	EBNA-1 EBNA-3C LMP-1 ^[63]	LANA-1 ^[64] v-CYC ^[65]	E6, E7 ^[66,67]	Naturally occurring pre-S mutants ^[68]	-	-
Lytic proteins	BZLF-1 ^[69] BGLF-5 ^[70]	-	E1, E2 ^[71,72]	HBx ^[73,74]	Core NS3 ^[23,62] NS5 ^[75]	Tax ^[76,77]

EBV: Epstein-Barr virus; HHV-8: Human herpesvirus 8, also named Kaposi sarcoma virus; HPV: Human papillomavirus; HBV: Hepatitis B virus; HCV: Hepatitis C virus; HTLV-1: Human T-cell leukemia virus 1.

Table 3 Virus products controlling cellular epigenetic modifications

Virus	EBV	HHV-8	HPV	HBV	HCV	HTLV-1
	EBNA-3A, EBNA-3C ^[78] LMP-1 ^[79] LMP-2 ^[80]	LANA-1 ^[81] microRNA ^[82]	E6 ^[83] E7 ^[84]	HBx ^[85,86]	Core ^[87]	Tax ^[88]

EBV: Epstein-Barr virus; HHV-8: Human herpesvirus 8, also named Kaposi sarcoma virus; HPV: Human papillomavirus; HBV: Hepatitis B virus; HCV: Hepatitis C virus; HTLV-1: Human T-cell leukemia virus 1.

members of different virus families can induce chromosome damage in infected cells *in vitro*^[15], and chromosome breakages have been observed in leukocytes isolated from patients experiencing systemic viral active infections^[16,17]. In recent years evidence has accumulated indicating the ability of different viruses to induce aberrant mitosis, genetic instability and interfere with cellular DNA repair pathways, which has confirmed early reports^[18-21]. Recent data suggest that viruses induce permanent damage in the genome of infected cells in the context of their natural infection^[22,23], and are capable of chromatin manipulation and epigenetic reprogramming of host expression patterns^[24,25]; it remains to be seen whether this could stimulate tumor initiation.

It is well known that viruses can transform non-permissive cells and several human viruses cause tumors if introduced in experimental animals. Interestingly all of the six human oncogenic viruses are able to establish latent and persistent infections (Table 1). Chronic HBV, HCV and EBV infections, persistent infection with HTLV-1, prolonged exposures or frequent reactivations of HPV and HHV-8 associated with clinical conditions, are all epidemiologically linked to increased risk of developing virus related malignancies^[26-32]. Failure to eliminate emerging tumor cells because of impaired immune function alone cannot account for this increased risk, since tumors develop in a minority of immune depressed patients. Furthermore tumor cells emerge very rarely from *in vitro* virally transformed cell lines, growing in the absence of immune selective pressure^[33]. When they do, these tumors are not associated with genetic instability^[34]. Therefore there is a missing causative factor acting in the setting of persistent infections, generally thought of as non viral carcinogens or host responses^[35]. We propose that reiteration and severity of infections/reactivations is a key factor that possibly generates primary genetic and epigenetic damage on which viral oncogenes may add up their own oncogenic activities.

A MECHANISM FOR TUMOR INITIATION IN VIRAL PERSISTENCE

Viral persistence can be achieved by continuous replication, latency or both. Several virus-encoded products have been associated with transforming and/or oncomodulatory activities^[4], and with the ability to induce chromosome damage, abnormal mitosis and genetic instability when expressed in cell cultures (Table 2)^[18,36-38]. Recent findings point to viral proteins interfering with the epigenetic milieu of the infected cells, leading to the transcriptional repression of tumor suppressor genes, and interference with cell cycling control^[39] (Table 3). However *in vivo* these activities must be particularly inefficient if one considers that the majority of the human population carries a number of resident viruses, but only a minority among infected individuals will develop tumors that can be correlated with persistent viral infections, and generally after very long latency periods (years to decades)^[4,35]. It should be noted that latency is characterized by a relatively low viral transcriptional rate^[40,41], that one can define as “a virus at rest”: this could explain why damaging and/or destabilizing activities of latent gene products have little chance to induce permanent effects in cells equipped with an intact set of caretaker genes, antioncogenes, and non activated oncogenes. Consistently, subjects with Fanconi’s anemia, an inherited disease with defective DNA repair, have up to 4000 times increased risk of developing solid papillomavirus-associated tumors^[4]. In fact cell immortalization has been achieved experimentally only following expression of latent genes in the context of previously accumulated mutations in the cellular genome^[18,20]. On the other hand, lytically infected cells are typically characterized by massive transcription of the viral genome, a “hit”. These cells develop virus induced chromosome damage and can undergo abnormal mitosis (Table 2), both *in vitro* and

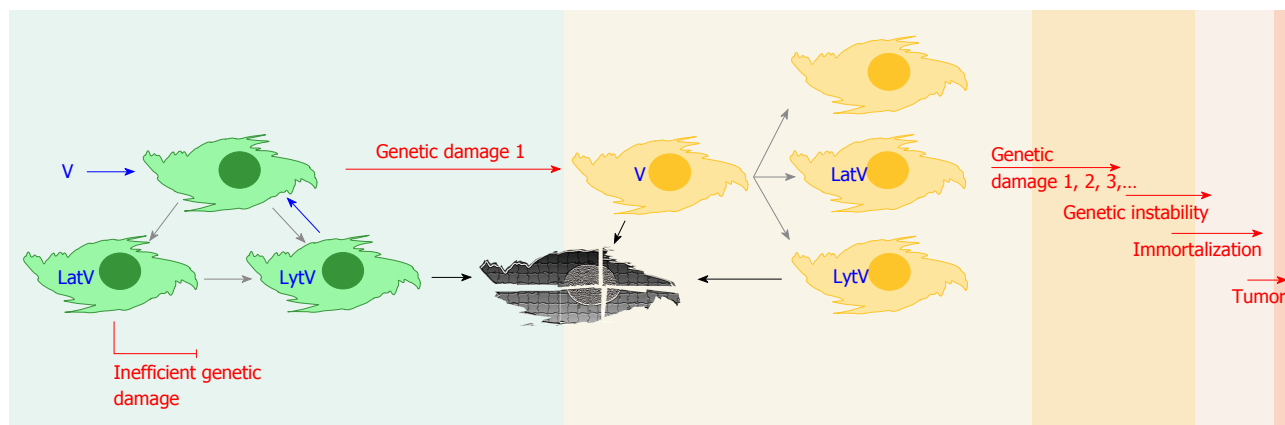


Figure 2 Tumor initiation events mediated by virus induced genetic damage. Virus entry into permissive genetically intact cells (green cells), can result in lytic replication or latency. In the latter setting the oncogenic potential of latent genes appears ineffective *in vivo*. Before silencing of most virus specific transcription is achieved, various viral functions are expressed which could induce genetic/epigenetic damage in a fraction of the infected population (red arrows, genetic damage 1). Cells surviving to sustainable damage (orange cells) could experience reactivation of the virus, host the virus genome in a latent state, or lose it after uneven segregation of their genetic material. In damaged cells latent gene products could now represent an effective oncogenic threat if cellular caretaker genes have been affected (red arrow, genetic damage 1, 2, 3...). Reinfection or reactivation of latent virus in damaged cells could result in further genetic offense, eventually leading to genetic instability, immortalization and tumor development. V: Virus; Lat: Latent; Lyt: Lytic. Blue arrows: Infection; Grey arrows: Consequences of infections; Black arrows: Death.

in vivo^[16]. So here we have two observations where there is apparently little if any effect on the genetic stability of healthy cells *in vivo*: (1) latency functions can transform cells but cause genetic instability only in already genetically damaged cells; and (2) lytic functions may induce genetic instability but kill the cells. Is there a setting in which these two phenomena may lead to an outcome that has been overlooked? The phase immediately following virus entry into a permissive cell, before the fate of the infection (lytic or latent) is set (green cells in Figure 2), may be crucial in this regard.

Latency is defined as an infection where the production of infectious virus does not occur immediately but the virus retains the potential to initiate productive infection at a later time, and is characterized by a unique transcriptional and translational state of the virus, the latency expression program, in which the productive replication cycle is not operative^[4]. Hence latency can be regarded as a transitory state of resistance of the infected cell to a virus, and the latency program as the result of a negotiation between virus and host, after a battle between cellular functions reacting to the incoming virus and virus encoded functions, expressed at early stages following virus entry into the host cell. The first consequence of this definition is that latency is not a default life program for a virus, but a survival condition that a virus is forced to opt for when the infected cell does not allow progression of the lytic cycle. A second consequence is that there is not one strictly planned latency program for any given virus, but the latency program is defined depending on the context of host cell gene expression, after the cell succeeds converting a viral hit into a virus at rest by resisting to the initial round of lytic cycle gene transcription, and forces the virus into the latency state, for some time. This state of resistance can last for very different periods of time, depending on the moment viral reactivation will be allowed by the infected cell, and can be long lasting, when

reactivation occurs upon transition into a new cell differentiation state, or last an unpredictable period of time, as in cells entering a particular metabolic state triggered by an infrequent external signal (ultraviolet radiations, stress, *etc.*), or cells being in a particular phase of the cell cycle^[42], which could be a frequent event for rapidly replicating cells or a very infrequent event for slowly replicating cells, as for liver cells. Recent evidence reveals that in an EBV latency model lytic genes can be transcribed to considerable levels^[43], contrary to what had been thought previously. Similarly in Kaposi sarcoma virus associated tumors, subpopulations of cells express lytic gene products within a general latency setting^[44], suggesting the distinction between latency and lytic transcription is less clear cut than expected. But what happens between viral entry into a cell and the establishment of latency in that cell? Very few studies have addressed this issue, but available data indicate that during this time lapse the majority of the viral genome is transcriptionally active, with many lytic genes being expressed in very much the same way as during early phases of lytic infection, before transcriptions are silenced by the host cell^[45,46]. This delicate, vastly unexplored resistant period may represent a particularly vulnerable setting for the infected host, acting as a non-permissive cell, a well known target for virus transformation^[1,47]. Therefore the actual phase between virus entry and the establishment of latency is a stage where some viral genes, whether belonging to the latent or the lytic expression program, can be expressed to various levels. Additionally, this is a time where the structure of the incoming virus disaggregates within the cell, releasing dozens of structural proteins and enzymes, genomic nucleic acids, coding and non coding RNAs, encapsidated in infectious particles. In fact it is now clear that the presence of incoming viral genomes relocates DNA repair proteins at sites of viral genome deposition^[48]. Several virus products are able to induce genetic damage (Table 2), and examples

of encapsidated DNA nicking activities with a potential role in chromosomal damage have been reported^[49,50]. Other viral proteins can interfere with the cellular DNA repair machinery (see^[51] for a detailed review) or introduce transcriptional-silencing marks^[39]. All these activities could in this context generate primary damage events, leaving the cell with permanent genetic and epigenetic damage before entering into latency. The ensuing latency program would now run in a cell bearing a modified genome.

Although it would be reasonable to expect that the majority of damaged cells could not survive the insult, it would be equally reasonable to expect that cells with sustainable damage may survive, as it is documented *in vitro* in non-permissive cells^[19,52] and in cells undergoing chemically induced DNA breaks and chromosome pulverization^[53].

A surviving cell could be imagined as acquiring a genotype with no phenotypic consequences on the virus, in which case the virus would either proceed with the lytic cycle and kill the cell or enter a latent state (rest), according to the virus and the type of infected cell (Figure 2). Alternatively genetic/epigenetic damage could modify the permissivity of the cell to the infecting virus, either further supporting viral expression programs or restricting them. The consequences on lytic infections would be either more productive lytic cycles or their inhibition with possible elimination of the virus, respectively. On latent infections the expression profile of the genome could be affected, either positively, as observed in EBV positive NK/T-cell lymphoma^[54], or negatively as it is observed when EBV latently infected B cells switch from the latency III (whole set of latency products expressed) to latency I (EBNA-1 only) following transformation into lymphoblastoid cells. Viral gene expression would now take place in the context of a genetically modified cell, and in some instances this combination could provide damaged cells with a selective advantage in their environment, making them fitter to survive such damage and ready for the accumulation of future genetic modifications, in other words placing them on the road to malignancy.

IS THE VIRUS LATENCY/REACTIVATION CYCLE AN ONCOGENIC THREAT?

While a single hit and rest event has little chance to set the stage for cancer initiation, repeated cycles of viral infection or reactivation and latency would increase the number of possibly genetically damaged cells in the host and eventually produce cells accumulating a number of chromosomal abnormalities, as recently observed in an *in vitro* model by Fang *et al.*^[55]. If the damage has modified or abolished the activity of caretaker genes, oncogenes or anti-oncogenes, then the genome damaging and/or destabilizing activities of viral latent gene products could now meet the requirements for the introduction of additional permanent damage, eventually leading to genetic instability. When the combination of hit and rest related damage

reached a critical point, let's say telomerase activation, the cell could become immortal and virus functions may become dispensable. Further damage due to genetic instability could lead finally to the emergence of a tumor cell (Figure 2). If the present hypothesis was confirmed, one consequence would be that the number of viruses with potential for tumor initiation would be larger than that currently accepted. A further consequence of the present hypothesis is that preventing virus reactivations, where possible by pharmacologic prophylaxis or medical modulation of the immune response, should counteract cancer development.

TESTING THE HYPOTHESIS

The demonstration that genetic and epigenetic damage occurs in latently infected cells and that some damaged cells survive in the setting of natural infections is crucial in validating our hypothesis. It would therefore be important to investigate the process whilst it is occurring. It is conceivable to plan prospective studies of patient populations at risk for recurrent or persistent viral infections. The genetic integrity of cells latently or persistently infected by a given virus could be studied using methods applicable to a large number of samples and correlated with virus shed at the site of sampling. For example the analysis of DNA damage could be associated with HPV isolation at the time of pap test screening, or with EBV viral load determination in the follow-up of transplant patients^[28]. Retrospective and prospective studies could be implemented, analyzing possible correlation between frequency of different virus reactivations, severity of these reactivations, evidence of genetic damage in cells that harbor latent viruses and development of malignancies, in order to better define the importance of evocative findings^[56]: ideal candidates for these studies would be populations of immunocompromised patients such as those in post-transplant settings^[57]. Chronic infections, clinically manifest or subclinical, are an additional interesting condition for virus related DNA damage investigation^[57]. In this setting the measurement of chromosomal abnormalities in peripheral blood lymphocytes should result particularly fruitful, if one considers that circulating cells are exposed to infectious agents even in localized infections during tissue perfusion^[23,58,59].

Precious information would be generated through the analysis of pre-tumoral and tumoral banked samples, where the observation of abnormal mitosis and genetic abnormalities can be associated with the identification of virus related antigens or nucleic acids^[60], while prospective studies could include virus isolation. As an example, hepatic biopsies from non-responders to anti-HCV treatment could be analyzed for the presence of genetic abnormalities and the findings would be compared to responders in relation to incidence of hepatocarcinoma development over time. *In vitro* studies should be devised choosing experimental settings that guarantee the closest simulation of authentic *in vivo* situations, cautiously choos-

ing animal models and transformed cell lines, avoiding non human cell cultures, and laboratory strains of viruses^[61]. Ideally fresh clinical virus isolates should be used to infect cells that are the authentic sites of latency *in vivo*, looking for consequences of virus infection on mitosis, chromosome integrity and the epigenetic stage.

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