Viruses in cancer cell plasticity: the role of hepatitis C virus in hepatocellular carcinoma

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Viruses are considered as causative agents of a significant proportion of human cancers. While the very stringent criteria used for their classification probably lead to an underestimation, only six human viruses are currently classified as oncogenic. In this review we give a brief historical account of the discovery of oncogenic viruses and then analyse the mechanisms underlying the infectious causes of cancer. We discuss viral strategies that evolved to ensure virus propagation and spread can alter cellular homeostasis in a way that increases the probability of oncogenic transformation and acquisition of stem cell phenotype. We argue that a useful way of analysing the convergent characteristics of viral infection and cancer is to examine how viruses affect the so-called cancer hallmarks. This view of infectious origin of cancer is illustrated by examples from hepatitis C infection, which is associated with a high proportion of hepatocellular carcinoma.

Key words: viruses, cancer cell plasticity, HCV, hepatocellular carcinoma.

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Introduction

It is estimated that close to 20% of human cancers are due to infections with known pathogens, mainly with viruses [1]. Given the difficulties in unambiguously assigning their causative role [2] and the fast rate of discovery of new viruses [3], it is likely a conservative estimate.

Different pathogens are associated with increased cancer risk. One of the first identified cancer-causing infectious disease was schistosomiasis, initially proposed in the middle of the nineteenth century by Theodor Bilhiarz to cause liver cancer and later shown to be associated with bladder cancer [4]. A bacterial infection with *Helicobacter pylori* is a risk factor for gastric ulcers and stomach cancer [5]. However, the main culprits among the infectious causes of cancer both for man and for animals are viruses.

Currently six viruses are classified as "carcinogenic to humans" by the International Agency for Research on Cancer (IARC). These are the high-risk human papilloma viruses (HPV), associated with quasi 100% of cervix uteri tumours and a significant proportion of anal, penile, and oral carcinoma; hepatitis B (HBV) and C (HCV) viruses, together responsible for 80% of hepatocellular carcinoma; Epstein-Barr virus (EBV), a causative agent of Burkitt lymphoma, some Hodgkin lymphomas, nasopharyngeal carcinoma, and some gastric cancers; Kaposi sarcoma virus (KSHV), a herpes virus associated with Kaposi sarcoma and human T-cell leukaemia/lymphoma virus type 1 (HTLV-1) that causes adult T leukaemia/lymphoma [1]. A recently discovered polyoma virus, the Merkel cell virus (reviewed in [6]), will probably join this list. Finally, HIV, while not directly oncogenic, is associated with cancer through the immunosuppression it provokes. Interestingly, immunosuppression can increase cancer risk either through disabling immune cancer surveillance or through preventing the host from controlling infections. Thus, AIDS aggravates cancer risk of HPV, HCV, EBV, and KSHV [1].

Human oncoviruses

Although the list of recognised human oncogenic viruses is surprisingly short, they are associated with some very frequent tumours. In fact, the conservative estimate is that 12% of global cancer incidence worldwide is caused by just four viruses (HPV, HBV, HCV, and EBV) [7, 8]. Moreover, it is an open question whether endogenous human retroviruses (HERVs) and retrotransposons are also cancer causing agents (recently reviewed in [9]).

Importantly, for all human oncogenic viruses, infection is much more frequent than tumour occurrence. In other words, only a small proportion of infected individuals will develop cancer. In fact, some of the oncogenic viruses are extremely widespread in the human population, the most striking example being EBV, for which serological analyses in diverse human populations show 80–100% seroconversion among adults [10]. With such a high

prevalence it is not intuitively obvious what it means to say that EBV causes several types of cancer. Indeed, establishing the causal relation between viral infection and cancer is not an easy task [2, 6], and stringent criteria have been proposed for such causality. A set of criteria was suggested by Hill 50 years ago [11] and is still widely used. It is a mixture of epidemiological and experimental points that can be summarised as follows (after [2]):

1) strength of association (how often is the virus associated with the tumour?),
2) consistency (has the association been observed repeatedly?),
3) specificity of association (is the virus uniquely associated with the tumour?),
4) temporal relationship (does virus infection precede tumorigenesis?),
5) biologic gradient (is there a dose response with viral load?),
6) biologic plausibility (is it biologically plausible that the virus could cause the tumour?),
7) coherence (does the association make sense with what is known about the tumour?),
8) experimental evidence (is there supporting laboratory data?).

In addition to meeting the epidemiological criteria, the knowledge of EBV biology strikingly reveals that different types of cancer are associated with alternative viral latency stages ([12] and references therein), providing unequivocal proof of causal relation between the two events. It should be noted, however, that some human oncoviruses (e.g. HTLV) have been classified as such on the basis of fulfilling only a subset of these criteria.

A historical perspective

The first demonstration of a virally induced tumour dates from 1908, when Ellerman and Bang described that avian erythromyeloblastic leukaemia could be transmitted by cell-free filtrates. The transmissible agent was later identified as avian myeloblastosis virus (AMV), encoding an oncogene *v-myb*. Soon afterwards, Peyton Rous reported similar experiments identifying the first case of a virus causing a solid tumour, again in birds: Rous sarcoma virus (RSV) encoding *v-src*. It took over 30 years to describe the first virus that caused tumours in rodents: mouse mammary tumour virus (MMTV) was shown to be transmitted by milk (Bittner agent). Interestingly, endogenous MMTV sequences are responsible for high predisposition to mammary tumours in some inbred laboratory mouse strains. Soon after the discovery of MMTV, a large number of murine leukaemia viruses (MuLV or MLV) were discovered in the 1950s (reviewed in [13]).

Remarkably, all of the early identified oncoviruses were retroviruses. Some carry a viral oncogene, the expression of which is necessary for transformation, but dispensable for viral replication and spread. In fact, the highly transforming retroviruses of this "acute transformation" group tend to be replication defective; they rely on helper viruses for their propagation. Interestingly, all replication-competent "helper" MLV can be oncogenic through insertional mutagenesis. The study of retroviruses gave rise to several 'change of paradigm' findings, several Nobel prizes, and unparalleled hopes for rapidly finding both the cause and the treatment for human cancers. The latter promise has clearly not been fulfilled: we now know that human cancer, even when linked to infection, is considerably more complex than suspected half a century ago. However, the seminal discoveries made in the field of oncogenic retroviruses set new bases for understanding cellular and molecular biology as well as cancer. Without any doubt, the most conceptually ground-breaking of these discoveries was the demonstration that cellular DNA encodes sequences homologous to viral oncogenes [14]; these "proto-oncogenes" [15] are essential for normal cell proliferation and function and some are deregulated in each occurrence of cancer [16].

The existence of cellular proto-oncogenes also explains the mechanism of transformation by non-acute retroviruses, which do not encode strong viral oncogenes [13]. Indeed, retroviral replication includes an obligatory step of reverse transcription of viral RNA genome and its more or less random insertion into the host genome. It follows that sometimes the proviral sequences, which include transcriptional regulatory regions in the long terminal repeats (LTRs) that flank the coding sequence, will be inserted in the vicinity of a proto-oncogene, the expression of which may become deregulated by this event, thus providing an initial trigger for cancerous transformation.

Attractive as the understanding of retroviral biology may be for describing the origin of cancer, so far only a single human retrovirus (HTLV1) is recognised as causative of a human cancer, and a rare one at that (adult T cell leukaemia/lymphoma) [17].

In sharp contrast to animal cancer, no human oncogenes have apparently been picked up by retroviruses, and insertional mutagenesis has only ever been observed in humans as a result of retrovirus-based gene therapy [18]. Several reasons can be evoked to explain this surprising difference between animal and human hosts. First, mass expansion of inbred lines of mice and chickens probably led to a selection of cancer susceptibility. Second, like other large and long-lived animals, humans have presumably evolved particularly efficient mechanisms to limit tumorigenesis, at least prior to post-reproductive age [19].

Historically, the first unambiguously identified human oncovirus is the EBV, a large dsDNA virus of the family *Herpesviridae*. Its oncogenic mode of action in the Burkitt lymphoma, where it was originally described, is as a necessary co-factor increasing survival of cells that have undergone an activating re-arrangement of the proto-oncogene *c-myc*. Interestingly, alternative viral genes and functionalities are involved in EBV oncogenic activities in other tumour types (reviewed in [20]). Other human oncoviruses belong to *Hepadnaviridae* (HBV), *Flaviviridae* (HCV), *Papillomaviridae* (HPV), and *Polyomaviridae* (Merkel cell polyoma virus – MCV). They are thus very different from one another and adopt different strategies to ensure their replication and spread. Nevertheless, common features of these distinct viral strategies, and the response they elicit from the host, can be identified. Elucidation of evolution-

ary adaptations of host-pathogen interactions has already unveiled, and will doubtless unveil in the future, important aspects of the biology of infection-related cancers and, more broadly, of cancer in general.

Viral strategies and cancer risk

Viruses adopt a plethora of strategies to ensure their propagation and spread. Some integrate their genetic material into the host DNA, either as a mandatory or as an optional step in the viral cycle. The integrated provirus replicates as part of the host genome and can become transcriptionally silent during a long latency phase. Latency can also be achieved by episomal maintenance of the viral genome; in this case some viral proteins are produced, and it is interesting to see that different subsets of EBV proteins characteristic of different latency stages give rise to distinct alterations of host cells and cause distinct types of tumours [12].

Viruses being obligatory parasites, all viral infections, be it acute, chronic, productive, or latent, hijack cellular machineries and lead to physiopathological changes of the infected cell. However, to the best of our knowledge, there are no convincing examples of oncogenic transformation in humans that are advantageous for the viral spread. So what are the mechanisms underlying the selection of viruses that cause cancer? We will argue that perturbations of cellular, tissular, and organismal homeostasis, which have evolved to ensure viral spread, make cells fragile so they become at risk of becoming cancerous. From this perspective, virus-induced cancer is a mere side effect of infection.

Whereas selective pressures that ensure optimal fitness operate on the level of individual virions, the situation is more complicated in most life forms. Indeed, in more complex organisms, including prokaryotes that typically live in colonies of quasi-identical individuals, the reproductive fitness of a cell is tempered by selective pressures operating on the level of cooperating cellular populations [21, 22]. The evolutionary passage to multicellularity, which brought advantages associated with increased size and tissular specialisation, required further harnessing of individual cells' survival and reproductive interests for the benefit of the organism as a whole. It can be argued that one consequence of multicellularity is the intrinsic risk of cells escaping the social controls exercised by other cells that are necessary for the harmonious function of the organism [23]. The escape from social controls leads to acquiring the propensity of uncontrolled growth and spread, i.e. the cancer phenotype.

A further paradigm for cancer development that is rapidly gaining support postulates a mandatory role for cancer stem cells (CSC). This concept states that tumour growth, spread, and resistance to treatments are driven by a subclass of tumoural cells with characteristics of stem cells, notably unlimited self-renewal (see [24] and articles in this special issue). So far, only few studies have addressed the link between viral infection and cancer stem cells. Others and we have shown that viruses can induce EMT (epithelial-to-mesenchymal transition), a process that may be instrumental in the acquisition of cancer stem cell phenotype [25, 26]. Furthermore, some viruses, for example HCV and EBV, activate the Hedgehog pathway, a key regulator of CSC phenotype [27–29]. Thus, it is very tempting to speculate that viruses have a role in this key carcinogenic event, although more work is needed to test this hypothesis.

In the following section we will use examples from HCV infection to illustrate how the viral strategies for reproduction can direct the infected cell and its microenvironment into loosening, or losing, their obedience to organismal interests and putting the cell on the path of oncogenic transformation.

Hepatitis C virus infection and cancer hallmarks

Hepatitis C virus (HCV) currently infects close to 150 million people worldwide, who are at risk for life-threatening liver pathologies, including a high incidence of hepatocellular carcinoma several decades after infection [30]. Hepatitis C virus is a small (about 9.4 kb) single strand non-integrative RNA virus that encodes a polyprotein that is cleaved into three structural and seven non-structural viral proteins. The narrow host range (man and chimpanzee) and inability of the vast majority of viral isolates to infect cultured cells made this virus particularly hard to study. However, relatively recent developments of sophisticated culture systems, transgenic mouse lines, and infectious models [31–36] significantly improved our understanding of HCV biology and the physiopathological consequences of the infection.

Like any other virus, HCV needs to recognise and enter its host cell, release and translate its genetic information, replicate its genome, and assemble and release infectious virions, which in this case are lipoviroparticles [37]. Two envelope proteins, E1 and E2, recognise several receptors present at the surface of the host cell, following which the virus is internalised by clathrin-mediated endocytosis. Upon release from endosomes, genomic RNA is translated via a cap-independent mechanism, and the resultant polyprotein is processed by proteases. Mature viral proteins are localised on endoplasmic reticulum and on ER-derived lipid droplet-associated vesicles called a membranous web, which is the site of genomic RNA replication. This is followed by virion assembly and release through the secretory pathway. Mobilisation of the host cell into virus production profoundly alters its physiology and its surrounding microenvironment. To analyse these changes in terms of risk of cancerous transformation, it is helpful to look at them in the context of the so-called cancer hallmarks, i.e. functional alterations characteristic of cancer cells [38, 39], depicted in Figure 1.

Hepatitis C virus infection exerts two major types of pro-oncogenic effects on the liver: direct effects of the viral gene products on hepatocyte physiopathology and indirect effects driven by sustained hepatic inflammation [40]. The former reflect viral strategies for exploiting the cellular machinery for the purposes of viral replication, and the latter correspond mainly to a response of the host aimed at viral clearance. As for other types of cancer, the

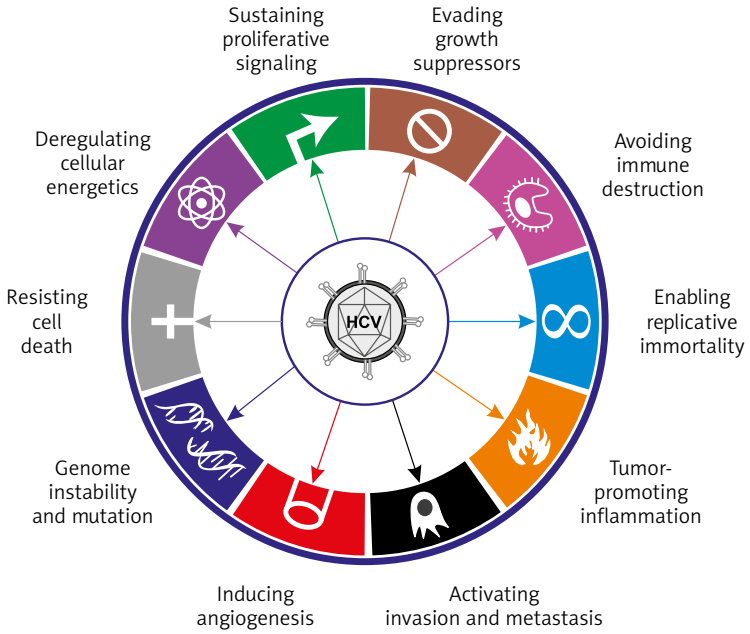

Fig. 1. Hepatitis C virus impacts all identified hallmarks of cancer. Modified with permission from [39]

resulting chronic inflammation is a major risk factor of carcinogenesis [41]. Interestingly, as discussed below, even the inflammatory signalling, the primary function of which is to eliminate the virus, appears to be exploited by HCV to improve its replication efficiency.

The first step of infection, the entry of HCV into a hepatocyte, is strictly dependent on several surface receptors, including occluding and claudin, a components of tight junctions, whose integrity is essential for epithelial architecture and function. While it is not known how the viral particle reaches these receptors, we and others have shown that several viral proteins, namely core (C), envelope (E1 and E2), and NS5A, use distinct molecular mechanisms to perturb hepatocyte polarity [42–44]. In consequence, viral proteins, often acting in concert with additional external or cell-autonomous stimuli, weaken cohesive forces that limit epithelial cells' motility [45]. In the most extreme cases they can trigger a full-blown epithelial-to-mesenchymal transition (EMT), a process through which epithelial cells acquire mesenchymal characteristics, displace the tight and adherens junction components and increase cellular motility and invasion [46]. This is indeed the case for hepatocytes expressing NS5A. Moreover, the viral protein cooperates with additional oncogenic stimuli to give rise to highly motile, invasive cells both *ex vivo* and *in vivo* [44]. Thus, the viral need to enter the cell is met by processes that activate **invasion and metastasis** and possibly acquisition of cancer stem cell phenotype. An alternative EMT induction pathway, triggered by E1/E2 proteins, employs TGF-β and VEGF signalling [43], thus impacting two additional cancer hallmarks: **evading growth suppressors** and **inducing angiogenesis**.

A major threat to a virus comes in the form of host defence mechanisms, both cell-autonomous and systemic, i.e. activation of programmed cell death and anti-viral immune response, respectively. Strikingly, similar pitfalls await a cancer cell [47]. Hepatitis C virus counters host cell apoptosis by several mechanisms, including possible interference with p53 signalling [48], activation of PI3K/AKT signalling [49], and direct interference with the apoptotic machinery, as exemplified by calpain-mediated proteolysis of Bid, a pro-apoptotic member of the Bcl2 family [50, 51]. Interestingly, the latter has a direct impact on the capacity of activated cytotoxic T cells to destroy hepatocytes harbouring viral proteins. This, in conjunction with molecular mimicry, such as alleviating the efficiency of antigen presentation [52], allows HCV-infected cells to **resist cell death** and **avoid immune destruction**, thus touching on two additional cancer hallmarks.

Metabolic alterations are another feature shared by the virus and a cancer cell. The much acclaimed Warburg effect, which is the heavy reliance of cancer cells on the glycolytic pathway even in the presence of oxygen (aerobic glycolysis), is in fact only one manifestation of altered cancer cell metabolism [53], which includes massive utilisation of glutamine and strong increase of lipogenesis, essential for macromolecular synthesis of fast-growing tumour cells. As already mentioned, HCV has a vital requirement for lipid droplets. Not surprisingly, therefore, chronic hepatitis C is frequently accompanied by lipid accumulation in the liver (hepatic steatosis). While the molecular mechanisms employed by the virus to switch on *de novo* lipid synthesis are not fully elucidated, recent work from our laboratory uncovered HCV-driven systemic modification of metabolic hepatocyte function [54]. Our data from transgenic mice and human clinical samples indicate that HCV triggers wnt/β-catenin signalling that alters the normal pattern of metabolic liver zonation, thus affecting both lipid and glutamine **metabolism**.

The remaining three hallmarks, namely enabling replicative immortality (i.e. avoiding senescence), genome instability, and tumour-promoting inflammation, have mixed cell-autonomous and micro-environmental origins. Chronic inflammation is a classical feature of unresolved, chronic infection, which is a typical feature of hepatitis C.

Its effectors are non-parenchymal cells recruited to infected livers. Others and we have recently shown that the initial triggers for such recruitment are small RNAs of cellular origins synthesised by NS5B, the viral RNA dependent RNA polymerase [55, 56]. These RNAs are recognised by intracellular Pattern Recognition Receptors that trigger intracellular **inflammatory signalling** that culminates in the synthesis and secretion of chemokines and cytokines, including lymphotoxin β, previously associated with murine and human hepatocarcinogenesis [57]. A question arises regarding the evolutionary advantage for a virus to use a polymerase that, in parallel to replicating viral RNA, triggers an anti-viral inflammatory response. There is at present no clear understanding of this issue, although it has been reported that both NF-κB and lymphotoxin are needed to maximise the efficiency of viral replication [58].

Once initiated, hepatic inflammation has profound effects on the liver microenvironment. It produces mutagenic reactive oxygen species, but first and foremost it activates hepatic stellate cells that secrete excessive amounts of extracellular matrix, leading to progressive liver fibrosis, culminating in cirrhosis. This is accompanied by hepatocyte death, which in turn leads to sustained regenerative activity within cirrhotic nodules, i.e. **sustained proliferation.** In consequence, there is telomere shortening in proliferating hepatocytes. Shortened telomeres are highly mutagenic and, in fact, **genomic instability** has been described during regenerative activity of cirrhotic liver [59]. Interestingly, hepatocytes within the regenerative cirrhotic nodules frequently undergo reactivation of telomerase [60], thus escaping the risk of chromosome breakage due to disappearing telomeres, but acquiring **replicative immortality**, a major cancer hallmark.

Of note, several cancer hallmarks that are initiated in chronic hepatitis C affect both the infected and the virus-free neighbouring cells: systemic alteration of metabolic pathways, inflammation, genomic instability, and replicative immortality are among those. One consequence of this effect is that both infected and neighbouring cells become targets for initiation of tumour growth. In agreement with this prediction, some tumours that develop on the background of hepatitis C cirrhotic livers contain high viral titres while others are practically virus-free (DG, unpublished).

This short review of common features between virus-imposed modifications of cellular physiology and common characteristics of cancer cells highlights the idea that HCV impacts all identified cancer hallmarks (Fig. 1). If so, why does it typically take several decades following infection to develop hepatocellular carcinoma? A plausible explanation is that hallmarks should be viewed as quantitative traits rather than "all or nothing" phenomena. Just like any other risk factor, HCV infection increases the probability of cancerous transformation. The estimated rate of hepatocellular carcinoma development on HCV infected cirrhotic liver, which is in the range of 3–7% annually, suggests that it does so very effectively.

New efficient drugs targeting HCV (the DAAs for direct acting anti-virals) have been developed [61]. When, and if, the issue of their scandalous cost is resolved, leading to their wide availability, they should limit the incidence of hepatocellular carcinoma, currently the fifth most frequent and the third most deadly cancer worldwide [62]. This note of cautious optimism notwithstanding, further study of virus-induced cancers in general and of the pathological consequences of hepatitis C in particular will doubtless continue to provide ever deeper understanding of the mechanisms of carcinogenesis and thus contribute to the development of novel, efficient, personalised cancer therapies.

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