REVIEW ARTICLE

Conceptual models for the initiation of hepatitis B virus-associated hepatocellular carcinoma

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Abbreviations
anti-HBc, antibodies to HBcAg; anti-HBe, antibodies to HBeAg; anti-HBs, antibodies to HBsAg; cccDNA, covalently closed circular DNA; CIN, chromosomal instability; CK19, cytokeratin 19; dsDNA, double-stranded linear DNA; ER, endoplasmic reticulum; HBcAg, HBV core antigen; HBeAg, HBV e antigen; HBsAg, HBV surface antigen; HBV, hepatitis B virus; HBx, HBV x protein; HCC, hepatocellular carcinoma; HPC, hepatic progenitor cells; hTERT, human telomerase reverse transcriptase; IU, international units; MAT−/−, methionine adenosyltransferase-knockout; MLL4, mixed-lineage leukaemia 4; NGS, next generation sequencing; pgRNA, pregenomic RNA; Pol, HBV DNA polymerase; rcDNA, relaxed-circular DNA; S/MAR, structural and matrix-attachment regions; TRAIL, tumour necrosis factor-related apoptosis inducing ligand; WHV, woodchuck hepatitis virus.

Abstract
Although chronic hepatitis B virus (HBV) infection is a known risk factor for the development of hepatocellular carcinoma (HCC), the steps involved in the progression from normal liver to HCC are poorly understood. In this review, we apply five conceptual models, previously proposed by Vineis et al. to explain carcinogenesis in general, to explore the possible steps involved in the initiation and evolution of HBV-associated HCC. Available data suggest that the most suitable and inclusive model is based on evolution of hepatocyte subpopulations. In this evolutionary model, HCC-associated changes are driven by selection and subsequent clonal expansion of phenotypically altered hepatocyte subpopulations in the microenvironment of the HBV-infected liver. This model can incorporate the wide range of mechanisms proposed to play a role in the initiation of HCC including oncogenic HBV proteins, integration of HBV DNA and chronic inflammation of the liver. The model may assist in the early prevention, detection and treatment of HCC and may guide future studies of the initiation of HBV-associated HCC.

Hepatitis B virus (HBV) is a blood-borne virus that causes significant morbidity and mortality worldwide. One-third of the world’s population has been exposed to HBV and ~400 million people are chronically infected (1). Chronically HBV-infected patients have a high risk of developing cirrhosis (2, 3) and a 25% cumulative life-
time risk of developing primary liver cancer in the form of hepatocellular carcinoma (HCC) (4). Cirrhosis and HCC generally manifest after several decades of chronic HBV infection, together causing the death of ~600,000 people annually (1). Unlike other causes of HCC where cirrhosis is almost ubiquitous, HBV-associated HCC has been reported to arise independent of other risk factors and occurs in ~30% patients of some ethnic groups (e.g. black males in sub-Saharan Africa) (5). Thus, the steps involved in the initiation and evolution of HBV-associated HCC appear to be more complex than those of other aetiologies and are not yet fully understood.

**HBV structure and replication**

Hepatitis B Virus is a small, enveloped, double-stranded DNA virus that contains four overlapping open reading frames, from which seven different proteins are translated. These include: three forms (large, medium and small) of HBV surface antigen (HBsAg); HBV core antigen (HBCAg); HBV e antigen (HBeAg); HBV x protein (HBx), that transactivates viral and cellular genes; and the HBV DNA polymerase (Pol), a viral reverse transcriptase that is essential for virus replication (6, 7).

The replication strategy of HBV is shown in Fig. 1. Firstly, HBV enters hepatocytes using the cellular receptor Sodium Taurocholate Cotransporting Peptide (NTCP) (8, 9). The HBV relaxed-circular DNA (rcDNA) genome is then transported into the nucleus where it is converted into covalently closed circular DNA (cccDNA) (C). cccDNA acts as a transcriptional template for mRNAs and pregenomic RNA (pgRNA) (D). pgRNA is encapsidated along with HBV Pol (black filled circles) into nucleocapsids composed of HBV core protein (E). Pol-mediated reverse transcription of the pgRNA occurs within the nucleocapsid resulting in the formation of either: 1) rcDNA (F) or 2) double-stranded linear DNA (dslDNA) (G). Nucleocapsids containing rcDNA and dslDNA are either cycled into the nucleus to add to the cccDNA pool or are enveloped and exported out of the hepatocyte as virions (H). The dslDNA that has entered the nucleus may integrate into the host cell chromosome (dotted lines) by non-homologous end joining (I).

**Fig. 1.** HBV replication. HBV (A) primarily infects hepatocytes (dashed rounded rectangle). After entering the cell via receptor-mediated endocytosis (B) through interaction between the HBV surface proteins (grey oval shapes) and the HBV receptor, Sodium Taurocholate Cotransporting Peptide (NTCP), HBV releases its double-stranded, relaxed-circular DNA (rcDNA) genome into the nucleus, where it is converted into covalently closed circular DNA (cccDNA) (C). cccDNA acts as a transcriptional template for pregenomic RNA (pgRNA) and mRNA (D). pgRNA is encapsidated along with HBV Pol (black filled circles) into nucleocapsids composed of HBV core protein (E). Pol-mediated reverse transcription of the pgRNA occurs within the nucleocapsid resulting in the formation of either: 1) rcDNA (F) or 2) double-stranded linear DNA (dslDNA) (G). Nucleocapsids containing rcDNA and dslDNA are either cycled into the nucleus to add to the cccDNA pool or are enveloped and exported out of the hepatocyte as virions (H). The dslDNA that has entered the nucleus may integrate into the host cell chromosome (dotted lines) by non-homologous end joining (I).

transcription, pgRNA and the HBV Pol enzyme are encapsidated in the cytoplasm by dimers of HBCAg to form a virus nucleocapsid. Within the nucleocapsid pgRNA is then reverse-transcribed by HBV Pol into either a rcDNA genome (present in 90% of virions) or a double-stranded linear DNA (dslDNA) genome (6). The HBV DNA containing nucleocapsids are then either enveloped and exported from the cell or are transported to the nucleus where rcDNA is converted into cccDNA, forming a pool of up to 30 cccDNA copies per cell (6, 10). At low frequencies, dslDNA molecules that are transported into the nucleus integrate into the host cell...
The most important disease outcomes associated with chronic HBV infection are cirrhosis and HCC, which generally occur in the latter three phases, but particularly in the HBeAg-negative chronic hepatitis phase. Factors associated with an increased risk of HCC include: the age of the patient; serum alanine aminotransferase levels >2 times the upper limit of normal; HBeAg-positive status; alcohol consumption; and underlying cirrhosis (22, 23). Virological features including HBV genotype C, high serum HBV DNA levels (>2000 IU/ml), the presence of the basal core promoter mutations A1762T or G1764A and the Pre-S mutations C1653T, T1753, and the absence of the pre-core mutation G1896A have also been associated with increased risk of HCC (24, 25).

If HCC is detected in its early stages (solitary tumours ≤5 cm or two or three tumours ≤3 cm), it can be effectively treated by partial liver resection or liver transplant with a 5-year survival rate of ~70%. However, recurrence of HCC is common and occurs in ~60% of patients treated with partial liver resection (26) and ~15% of patients treated with liver transplant (27). Some factors that predict HCC recurrence include: de novo HCC arising from the remaining non-tumour liver; local metastases from the primary tumour and HBV-reinfection of the liver graft (28, 29). Because of the marked resistance of HCC to chemotherapy and radiotherapy (30), no curative therapies for HCC exist beyond these early stages.

Therefore, early diagnosis of HCC is central in improving survival outcomes. The currently used biomarker for HCC, serum alpha fetoprotein, has poor sensitivity and specificity in the early stages of HCC, as its expression is a relatively late, or even sometimes absent, feature of HCC (31, 32). This has stimulated research into the initiation of HCC with the hopes of developing biomarkers for the early stages of HCC.

## The cellular origins of HCC

While it has been assumed that HCC arises from the transformation of hepatocytes, some researchers have suggested that hepatic progenitor cells (HPC) can give rise to HCC (33). HPC are suggested to be long-lived bipotent stem cells that can differentiate into hepatocytes or biliary epithelial cells with the capacity to result in tumours with HPC-like expression profiles (33). In physiological conditions, HPC are located close to bile duct epithelial cells, they strongly express cytokeratin 19 (CK19), have scant oval-shaped cytoplasm and are slightly smaller than hepatocytes (34).

The presence of integrated HBV DNA in ~90% of HBV-associated HCC indicates that HCCs are generally derived from HBV-susceptible cells (35). Studies of HBV-infected liver from our laboratory suggest that CK19-positive HPC do not express HBV antigens, and do not support HBV replication. As shown in Fig. 2, HBsAg and CK19 were detected by dual immunofluo-
rescence staining of sections of HBV-infected liver. No CK19-positive cells that displayed scant oval-shaped cytoplasm and a smaller cellular size than hepatocytes were HBsAg-positive. However, rare HBsAg and CK-19 dual-positive cells were detected that displayed morphologies more similar to hepatocytes than HPC (Fig. 2). The HBsAg and CK19 dual-positive cells were located close to single CK19-positive cells, suggesting that the dual-positive cells were hepatocytes that had recently differentiated from HPC precursors. These results contradict a previous study that suggested that CK-19 positive HPC can support HBV replication (36), but are consistent with other studies that observed post-entry blocks in HBV replication in non-hepatocyte liver cells, including HPC (37, 38) and with recent fate-mapping experiments (39) that question

Fig. 2. Dual immunofluorescence staining of HBsAg and CK19. Two different fields of view of formalin-fixed liver from a HBV-positive patient with cirrhosis are shown in 4 panels on the left and 4 panels on the right. HBsAg was detected using an anti-HBsAg primary antibody and Alexa594-conjugated secondary antibody at 532–587 nm excitation and 608–683 nm emission. CK19 was detected using and anti-CK19 primary antibody and Alexa488-conjugated secondary antibody at 470–490 nm excitation and 520–560 nm emission. Nuclei were counterstained using DAPI (detected at 330–380 nm excitation and >435 nm emission). Merged images show that HBsAg and CK19 dual-positive cells (long, thin arrows) have a similar size and morphology to neighbouring hepatocytes, as opposed to single CK19-positive cells (short, thick arrows) with similar morphology to HPC. Magnification 60x. Scale bar = 25 μm.
the existence of liver stem cells. They are also consistent with in vitro studies showing that the human HPC line, HepaRG, must be differentiated to express NTCP, the receptor for HBV (9).

Potentially, the remaining ~10% of HCCs without integrated HBV DNA may represent tumours arising from HPCs. Alternatively, others have suggested that the loss of susceptibility of hepatocytes to HBV may be a survival advantage selected for in the liver microenvironment that predisposes the non-susceptible hepatocytes to the development of HCC (40, 41). Finally, as HBV integration is a rare event [estimated to occur once per 10 000 cells after 6 days of infection in the duck model of HBV(11)], random probability may allow HCC to arise from hepatocytes without integrated HBV DNA. Therefore, while the contribution of HPC to the origin of HCC remains controversial, we have taken the view that transformation of hepatocytes is the main contributor to HBV-associated HCC.

Models for the initiation of HBV-associated HCC

The different theories regarding the progression from normal liver to HBV-associated HCC can be categorised using the five different conceptual models, proposed by Vineis et al. (42) and used to explain carcinogenesis in general. While each of the five models focuses on a different aspect of the initiation and evolution of HBV-associated HCC, we accept that the models overlap considerably and should not be considered as exclusive. Furthermore, the models are generalised for clarity in discussion, although we acknowledge that our descriptions may not represent some subtleties in the various models and that these are not the only models discussed in the literature. Below, we review each of these five models and their proposed roles in the initiation of HBV-associated HCC.

The mutational model

The first conceptual model described by Vineis et al. (42) proposes that the main mechanism for carcinogenesis is the random mutation associated with cellular turnover [\(-10^{-8}\) mutations per nucleotide per cell division (43)]. While the majority of mutations that accumulate in this way are not associated with the carcinogenic process (termed passenger mutations), by chance some random mutations will occur in genes that cause progression towards cancer (driver mutations). Additionally, the chronic inflammation associated with HBV infection induces hepatocyte turnover, which enhances the rate of random mutation and so increases the risk of initiation of HCC. As mentioned previously, the HBV dsDNA can randomly integrate into the host cell genome by non-homologous end joining (12), potentially contributing to the increased cellular mutation rate via insertional mutagenesis. Indeed, HBV DNA integration into oncogenes, such as the mixed-lineage leukaemia 4 (MLL4) gene and human telomerase reverse transcriptase (hTERT) gene, have been observed using next generation sequencing (NGS) in HCC (44–46). The requirement for the accumulation of driver mutations over time resulting from chronic inflammation, increased cell turnover and insertional mutagenesis may explain the late onset of HCC that often occurs at 40–60 years of age, following exposure to HBV in childhood.

However, with the advent and application of NGS, this mutational model has been shown to be overly simplistic. In whole genome sequencing studies, cancers do not show a random distribution of mutations, but instead show at least 21 different mutational signatures categorised by the frequency of particular base substitutions (47). HCC has been found using whole genome and whole exome sequencing to be one of the most heterogeneous cancers with at least six different mutational signatures observed, reflecting a complex mutational process and not simply stochastic DNA mutation (46–49). Furthermore, in studies of chronic HBV infection, no cellular gene has been observed to be consistently disrupted by HBV DNA integration (44–46, 48–50), contrasting with studies of woodchuck hepatitis virus (WHV) infected woodchucks where the N-Myc gene is a common target for WHV DNA integration (51). Moreover, the integration of HBV into hTERT and MLL4 in non-tumour tissues cannot be discounted because NGS techniques such as whole genome or exome sequencing often fail to detect unique, low frequency, HBV DNA integrations present in smaller hepatocyte subclones. Therefore, the NGS studies that identified the hTERT and MLL4 hotspots (44–46) may contain biases for larger clones.

The simple model of HBV-associated HCC as a linear progression of mutations does not account for the multiple molecular changes associated with HCC: for example, altered expression of mRNA, miRNA and proteins (52). Among these, the most common pathways that are mutated in HCC include the Wnt/β-catenin-, Hedgehog-, p53- and JAK/STAT-dependent pathways (52). However, while there are alterations in expression in particular genes (e.g. hTERT, p16 and TP53) and pathways that drive HCC progression, these changes only occur late in tumour progression (53) and not in all cases of HCC (54, 55). This variation is due in part to the multiple subtypes of HCC that can be recognised by distinct transcriptional profiles (56). However, even when classified into molecular subtypes, the family of HCC tumours contains a high level of heterogeneity. This suggests that the initiation of HCC does not occur simply by accumulation of random DNA mutations in a linear sequence and requires a more detailed model to be fully described.

The genomic instability model

The second conceptual model described by Vineis et al. (42), the genomic instability model, extends the first by
suggested that the rate of mutation can be altered by various mechanisms, including chromosomal instability (CIN), microsatellite instability and focal hypermutation. Indeed, in mouse models of chemically induced HCC, DNA mutations associated with genomic instability predict susceptibility to the development of tumours (57). Furthermore, the majority of HBV-associated HCC have been associated with chromosomal rearrangements and copy number variations (46, 49).

In HBV infection, integration of HBV DNA leading to destabilization and mass rearrangement of the host-cell genome has been studied in detail (46, 58, 59). In many virus infections, DNA integration into the host cell chromosome induces CIN (60–63). In these cases, CIN can be induced by the integration into cellular genome sequences called structural and matrix-attachment regions (S/MAR), which modulate the activity of gene enhancers, interact with transcription-associated proteins, and are also associated with origins of cellular replication (64, 65). Virus DNA integration into S/MAR and the resulting CIN has been observed in HCC associated with chronic WHV infections (66) and HBV-infected cell lines (67). Thus, the association between HBV DNA integration and CIN has led to the speculation that CIN induces the initiation and progression of HCC (45, 54, 58).

However, integration of HBV DNA is not sufficient for HBV-associated hepatocarcinogenesis. Hepatocytes with normal histology have been observed with integrated HBV DNA, suggesting that integration into coding or non-coding DNA sequences does not necessarily induce changes in the phenotype of the host cell (35, 68). Indeed, Jiang et al. showed that the number of HCC driver mutations does not correlate with the number of unique HBV DNA integrations found in HCC (50). Furthermore, no significant differences in DNA rearrangement caused by HBV DNA integration, CIN, microsatellite instability or focal hypermutation were observed between tumour and non-tumour tissues (50). Other studies, however, directly contradict this observation (46) or show that levels of genomic rearrangement are highly variable in HCC-associated with HBV from different patients (69, 70). Thus, while playing a role in tumour progression, the evidence does not support a requirement for HBV DNA integration or genetic instability in the initiation of HBV-associated HCC.

The epigenetic model

This third conceptual model described by Vineis et al. (42), the epigenetic model, introduces the role of non-mutational molecular changes on the cellular DNA sequence. These include epigenetic DNA modifications and alterations in cellular signalling pathways by virus proteins. CpG methylation of cellular promoters silences downstream transcription (71). A wide range of cellular gene promoters have an altered methylation status in HCC.

Progressive hypermethylation in the promoters of the APC, GSTP1, RASSF1A and p16 genes have been detected during the progression from normal liver to HCC (72, 73). However, changes in DNA methylation are not sufficient for hepatocarcinogenesis: while promoters of the negative regulators of cell growth p16, HAL2 and RASSF1A were found to be methylated in 90% of tumours, the same methylation was also observed in 56% of neighbouring, histologically normal liver tissues (74).

The oncogenic potential of HBV gene products, particularly HBx and HBsAg, has been widely described (75, 76). While HBx is a necessary factor for HBV replication (77), its exact role during replication remains unknown. The major reported role for HBx is transactivation of host proteins, reportedly through which HCC is induced (59). For example, expression of HBx upregulates hTERT, and the vaso-invasion-associated genes HIF1-alpha and histone deacetylase 1, and increases degradation of β-catenin in primary human liver and models of HCC both in vitro and in vivo (78–81). Overexpression of HBsAg in transgenic mice also induces precancerous liver lesions and HCC in the majority of animals (82, 83), suggesting a direct oncogenic role. Moreover, endoplasmic reticulum (ER) stress-related responses resulting from expression of HBsAg with Pre-S mutations are associated with chronic HBV infection (84, 85) and an increased risk of HCC (25). ER stress has also been shown to be a crucial factor in the initiation of HCC in some mouse models (86).

However, while a large range of functions have been attributed to mutations in HBx and HBsAg, many of these studies which involve overexpression, can be criticized as not being representative of disease states. For example, in many studies the HBx gene was expressed under the control of a highly active CMV promoter (78–80). Furthermore, the development of HCC is not consistent with the natural history of HBV infection. During the early immune tolerant phase, high levels of HBV antigens are expressed and high titres of up to 2 × 10^9 IU/ml guarantee that some HBV genomes with Pre-S mutations exist in HBV quasispecies population in the host (16, 17). However, HBV-associated HCC generally does not develop until decades after the initial infection. While it may be that HBsAg with Pre-S mutations are more important in later phases when it is the major form expressed in the hepatocyte and accumulates in the ER (84, 85), the mechanism and extent of the role of HBsAg in HCC initiation still remains unclear. Together, these data suggest that epigenetic changes and HBV gene products, are not sufficient to initiate HCC.

The microenvironmental model

The fourth conceptual model described by Vineis et al. (42), the microenvironmental model, acknowledges that cells do not exist in isolation and are subject to ongoing local stimuli within their microenvironment to initiate and drive carcinogenesis. In particular, the chronic
inflammatory environment is virtually ubiquitous in all forms of liver cancer and in the precancerous liver and plays a major role in carcinogenesis (87). As described below, chronic inflammation associated with HBV infection may initiate HCC by (i) stimulating fibrogenesis, (ii) inducing an inflammatory cytokine milieu and (iii) increasing the exposure of hepatocytes to oxidative stress.

Chronic inflammation and hepatocyte death are known to contribute to progressive fibrosis and the development of cirrhosis (88). Cirrhosis-associated vascular disruption leads to disturbed blood flow in liver nodules, impaired nutrition and oxygen distribution to hepatocytes and to a subsequent cascade of cellular responses that lead to carcinogenesis (87). The hypoxia associated with cirrhosis induces a more tolerogenic phenotype in infiltrating and liver-resident immune cells (89), potentially allowing preneoplastic hepatocytes to escape immune surveillance (90). Furthermore, cirrhosis is associated with altered hepatic stromal cells that provide pro-oncogenic signals to surrounding hepatocytes (91). Indeed, a strong association exists between cirrhosis and HCC with 80–90% of patients with HCC from different aetiologies having concomitant cirrhosis (5). The observation that post-resection HCC recurrence occurs with regularity on the background of cirrhosis, despite antiviral treatment and removal of the original nodules of HCC, suggests that the liver microenvironment is already profoundly altered to give rise to a pro-carcinogenic milieu (92).

However, in selected populations with chronic HBV infections (e.g. black males in sub-Saharan Africa), HCC can occur without cirrhosis in up to 30% of cases (5). This suggests that cirrhosis, though a strong predictor and initiator of HCC, is not necessary for the initiation of HBV-associated HCC. Furthermore, cirrhosis is not sufficient for HCC, as evidenced by patients with HBV-associated cirrhosis having only a 5-year cumulative HCC risk of ~15%, with the majority of patients not progressing to HCC at all (5). Thus, HCC initiation and progression may be thought of as strongly related to, but can also occur independent of, cirrhosis (Fig. 3).

Inflammation-associated signals, such as growth factors, chemokines and cytokines, from non-hepatocyte cells have been postulated to alter the microenvironment of the chronically HBV-infected liver to a pro-oncogenic state. For example, the pro-inflammatory cytokine macrophage migration inhibitory factor has been shown to inhibit the transactivation properties of p53 (93). Additionally, stress-activated MAP kinases are upregulated in the liver during chronic inflammation and are involved in HCC-associated pathways (94). However, inflammatory signals alone are not sufficient to cause HCC as short-term inflammation associated with the clearance

![Fig. 3. A proposed model of HBV-associated liver disease.](image-url)
of transient HBV infection confers a low risk of development of HCC (95).

Oxidative stress associated with chronic HBV infection has been implicated in increasing genomic DNA mutation. Oxidative stress, such as that experienced by chronically inflamed liver, has been shown to increase double-strand DNA breakage in host cells (96). Many sources of oxidative stress, such as endothelial inducible nitric oxide synthase expression and reactive oxygen species produced by infiltrating immune cells, can be found in chronically inflamed liver (97, 98). DNA breakage, the substrate for HBV integration (12), especially occurs in base unpairing regions in the DNA genome, including S/MAR (99). Indeed, increased HBV integration has been observed in HBV-infected HepG2 cells under oxidative stress induced with hydrogen peroxide (100). Furthermore, methionine adenosyltransferase-knockout (MAT-/-) mice, which have increased levels of hepatic oxidative stress, display an increased rate of HCC (101). However, oxidative stress is not sufficient for the initiation and progression of HCC. Despite being more susceptible to the carcinogenic effects of carbon tetrachloride-induced liver damage, MAT-/- mice did not spontaneously develop HCC (101). Moreover, chronic oxidative stress is found in the liver of patients with Wilson’s disease as a result of copper accumulation, but the risk of HCC is lower in these patients (0.04% per year) than in chronic HBV infection (~3% per year) (5, 102–104).

The evolutionary model

The fifth conceptual model proposed by Vineis et al. (42) is based on Darwinian evolutionary theory. Several groups have used evolutionary models to integrate disparate aspects of the initiation, development and progression of cancer (42, 105–107). While it has been long-reported that evolution occurs within an established tumour (57), there is increasing evidence that selection, clonal expansion and evolution of populations of hepatocyte subclones occurs prior to tumour initiation. Indeed, extensive clonal expansion of hepatocytes has been observed not only in cirrhotic regenerative nodules (46, 50, 69, 108–110), but also in non-cirrhotic liver from patients with chronic HBV infection (46, 50, 68, 70).

In this model, there are increased rates of DNA mutation or epigenetic changes associated with HBV infection, as a result of direct mutagenesis through increased cellular turnover, HBV DNA integration and oxidative stress (Model 1); genomic instability (Model 2) and/or epigenetic changes in the cellular genome (Model 3). These factors cause phenotype variation within the hepatocyte population, on which natural selection acts within the microenvironment (Model 4). Selection for pro-oncogenic changes causes clonal expansion of hepatocyte subpopulations and promotes the initiation of HCC. The evolutionary model incorporates aspects of the previous four conceptual models and addresses some of their individual weaknesses. Though these explanations are largely theoretical, they are supported by the literature.

As described in Models 1 and 2, DNA alterations in HCC are not random, but instead show multiple mutational signatures (47). Loeb et al. has popularised the ‘mutator phenotype hypothesis’, where the cellular phenotype of increased DNA mutation rate is an inevitable selective advantage in the preneoplastic microenvironment (105, 111). This increased mutation rate may occur through different mechanisms, such as: losing DNA repair mechanisms; upregulation of the interferon stimulated gene APOBEC, a cytidine deaminase that induces hypermutation or increases in methylated CpG motifs (which are susceptible to C>T mutation). Though not one of these mechanisms appear in isolation to consistently lead to HCC, DNA hypermutation in general may be selected for in this evolutionary model and may drive the multiple mutational subtypes observed in HCC (47).

Non-mutational molecular changes associated with HCC (Model 3), such as CpG methylation and HBV gene product expression, occur in non-tumour hepatocytes and early in the HBV infection while HCCs occur late in HBV infection, suggesting that they do not have direct oncogenic roles in HBV-associated HCC. In this evolutionary model, CpG methylation and HBV gene product expression could instead be affecting evolution of the hepatocyte population leading up to HCC, e.g. by causing transient molecular changes that alter the evolutionary landscape, allowing cells to move more easily into more cancer-like quasi-stable states through stochastic perturbations (112, 113). If driven by CpG methylation, this state can be 'locked in' because of the susceptibility of methyl-cytosine to be converted into a thymidine (114). The role of pro-oncogenic alterations in promoting the evolution of tumour cells has been described by others in detail, though these theories have yet to gain significant traction in the field (112, 113, 115).

In this evolutionary model, the chronic inflammatory microenvironment (Model 4) can drive clonal expansion of hepatocytes and HCC in multiple ways. Firstly, a period of chronic inflammation may be required to create replicative space for preneoplastic hepatocytes to clonally expand and thus become more common in the hepatocyte population. As the liver maintains its size through multiple redundant cell-cycle checks (116), hepatocyte clones may only divide when compensatory mitosis is initiated after hepatocyte death. Furthermore, chronic inflammation may itself select for preneoplastic properties. For instance, tumour necrosis factor-related apoptosis inducing ligand (TRAIL) is highly expressed during inflammation and can induce apoptosis in HBV-infected cells (117, 118). Chronic exposure of the hepatocyte population to TRAIL may select for hepatocytes that can escape TRAIL-mediated apoptosis, e.g. through the loss of the TRAIL receptor, a common alteration in
HCC (119, 120). It may be by these mechanisms that chronic (but not acute) inflammation drives the evolution towards HCC.

Therefore, we propose the following steps for the initiation of HBV-associated HCC. These steps are also illustrated in Fig. 4:

- As a result of natural variation caused by random mutation and epigenetic factors, a small subpopulation of hepatocytes that have changes that confer a heritable survival advantage exist in the HBV-infected liver;
- Chronic inflammation causes the ongoing death of HBV-infected hepatocytes, freeing up replicative space allowing hepatocytes with a survival advantage to clonally proliferate.
- Hepatocytes with a heritable survival advantage undergo further molecular changes allowing selection of preneoplastic hepatocytes.
- Eventually, hepatocytes with preneoplastic changes initiate the development of HCC.

**Predictions and implications**

Importantly, the evolutionary model makes several testable predictions.

Firstly, if this model of cancer progression is correct, then the development of HBV-associated HCC occurs not only late in HBV infection, but also potentially...
throughout the course of chronic HBV infection. In HCC, numerous studies have shown that clonal expansion of altered hepatocytes is a risk factor for carcinogenesis (121). Clonal expansion of hepatocytes with an increased proliferation index compared to surrounding hepatocytes have been observed in a carcinogen-induced rat model of HCC (122, 123). Also, in patients with hereditary tyrosinaemia, clonal expansion of hepatocytes with mutations that correct for an inherited deficiency in fumarylcoacetase has been observed in the development of HCC (124). In the context of chronic HBV infection, previous studies have proposed that hepatocytes with a survival advantage could clonally expand in the environment of a HBV-infected liver (41, 68, 110, 125). While the specific survival advantage(s) have not been identified, Mason et al. have suggested that they could occur through an increased responsiveness to growth signals, loss of virus expression, failure of immune presentation of virus antigens or otherwise resisting immune-mediated cell death (41). Indeed, clonal expansion of non-tumour hepatocytes with a survival advantage has been observed in the liver of patients with chronic HBV infection, as well as in the chimpanzee and woodchucks models of HBV infection (41, 68, 110, 125). Furthermore, as we have shown in a previous study (126), clonal expansion of hepatocytes has been observed in the liver of patients at all stages of chronic HBV infection including in the immune tolerant phase, a period during which patients are not considered at risk of HCC. This clonal expansion could not be explained by random hepatocyte turnover and suggested that at least some of the clonally expanded hepatocytes had a survival advantage.

This implies that the risk of HCC accumulates not just in the later stages, but throughout chronic HBV infection as a continuous process before detectable histological changes are observed. Indeed, multiple independent studies show that histologically normal, peritumoural liver commonly display HCC-associated changes in protein expression, DNA methylation and mutation in oncogenes (74, 127, 128). Furthermore, mathematical models of the evolution of cellular clones in a self-renewing solid tissue (e.g. the liver) during the precancerous phase have suggested that >50% of driver mutations occur prior to tumour formation (129). In the current debate about antiviral treatment during the immune tolerant phase of chronic HBV infection (130–132), this model would predict that earlier antiviral treatment could slow down or prevent the progression of HCC. Conversely, antiviral treatment later in the course of chronic HBV infection may not reduce the incidence of HCC as the liver may already contain preneoplastic hepatocyte subclones that have arisen by clonal expansion. Indeed, this may explain the poor efficacy of nucleoside analogues in preventing the initiation of HBV-associated HCC in randomised clinical trials (133).

Secondly, the presence of these potentially preneoplastic hepatocyte subclones in the liver during late stage disease could be clinically relevant for treatment. Each of the subclones may have followed a separate evolutionary course owing to the local liver microenvironment or random chance and so would contribute to the heterogeneous nature of HCCs that are observed clinically. This clonal heterogeneity may be a contributing factor to the marked chemotherapeutic resistance of HCC (31). While it is tempting to posit that other evolutionary features [such as interclonal cooperation (134)] could even accelerate the initiation of HCC, no clear evidence has shown this to date.

Thirdly, selective advantages causing clonal expansion are subtle and, on average, cause a net increase in cell fitness in the order of 0.5% (135). Thus, unless they are specifically designed to detect these small changes, many in vitro experiments may not be sensitive enough to demonstrate the effect of suspected driver mutations. Furthermore, a selective advantage must be considered within the specific liver microenvironment. As such, a suspected selective advantage may not have been identified as it has been removed in cell culture conditions from its context (e.g. other cells, hypoxic conditions, cytokine milieu, etc.). While it is technically difficult to define and recapitulate these subtle conditions, this evolutionary model predicts that preneoplastic hepatocytes possessing a selective advantage are present and would clonally expand within this microenvironment.

A fourth testable prediction of this model is the requisite presence of clonal expansion of hepatocytes with altered genotypes and phenotypes leading up to HBV-associated HCC. Thus, altered clonal expansion could potentially predict the progression of the liver towards HCC. Measures of hepatocyte clonal expansion could theoretically detect all of the heterogeneous subtypes of HCC. Alternatively, isolation of large cell clones could aid in the identification of molecular changes that impart a survival advantage that ultimately leads to HCC. It may be more likely that these cells express potential biomarkers for early detection of HCC.

Intriguingly, clonal expansion of hepatocytes has been detected by our group and others in the non-tumour liver tissue of patients with HCC, regardless of cirrhosis (68, 126). This suggests that selective pressures associated with clonal expansion (and presumably with progression of HCC) are separate from chronic inflammation and associated liver injury (Fig. 3). This potentially explains the ability of HBV-associated HCC to arise without cirrhosis. Further studies are needed to characterise these selective pressures and to determine what drives HCC on cirrhotic and non-cirrhotic backgrounds. In patients with cirrhosis, HCC is likely to have evolved in a microenvironment with high levels of chronic inflammation, cell death and signals for compensatory cell division, making it likely that hepatocytes with a heritable survival advantage are able to evade immune-mediated cell death. Conversely, HCCs that have evolved in a non-cirrhotic background are selected.
in a liver microenvironment with lower levels of inflammation and growth factors, which may instead select for enhanced growth and a lack of cell-cycle checkpoints rather than immune evasion.

Finally, an evolutionary model may reveal potential new targets for HCC therapy. Evolutionary simulations and bioinformatics analysis suggest that cells with a survival advantage should contain a greater number of passenger mutations that give a survival disadvantage to the cell, so-called deleterious passenger mutations (136). The accumulation of these deleterious passenger mutations are hypothesised to be caused by two conditions: (i) that deleterious passenger mutations occur more frequently than driver mutations, and (ii) that the accumulation of deleterious passenger mutations eventually offsets the survival advantage of the driver mutations. McFarland et al. suggest that by increasing the effect of these existing passenger mutations by upregulation of the unfolded protein response pathway, the initiation, growth or recurrence of HCC may be reduced. Alternatively, other authors have suggested that the incidence of HCC can be reduced by altering the microenvironment to give more benign cellular clones a survival advantage, thereby outcompeting premalignant clones (137).

Conclusions

HBV infection is responsible for significant worldwide mortality through the development of cirrhosis and HCC. No single cellular pathway appears to be the initiator of HBV-associated HCC, so it is likely that a combination of molecular mechanisms in the context of the liver microenvironment drives hepatocarcinogenesis. We have discussed how the interplay between these mechanisms and the hepatocyte population can be viewed as an evolutionary model. This evolutionary model is consistent with our current knowledge of the initiation of HBV-associated HCC, but emphasises the role of the chronic inflammatory liver microenvironment and its effect on the clonal expansion of hepatocytes, selectively amplifying cells containing preneoplastic changes. We believe that applying these evolutionary concepts may assist in developing strategies for the early prevention, detection and treatment of HCC.

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