Molecular and cellular oncogenic mechanisms in hepatocellular carcinoma

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Hepatocellular carcinoma (HCC), as the fifth most diagnosed cancer in the world and the third leading cause of death, is a global health concern. Research stimulated by the dismal prognosis of HCC has led to significant advances in the understanding of its aetio-pathogenesis. Dysregulation of genetic, epigenetic and signalling pathways as well as tumour immunological escape mechanisms are implicated in the development of HCC. This review summarises the current knowledge of these mechanisms and argues that it is only through further understanding of their role in hepatocarcinogenesis, that new effective therapies can be developed.

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Hepatocellular cancer (HCC) is the most common primary cancer of the liver and was accountable for 782,000 incident cases in 2012. Of these cases, an alarming 745,000 patients died.1,2 These figures attest to the dismal post-diagnosis outlook (3.4 months median survival) with or without screening.2,3 The chief risk factor for HCC in most patients is cirrhosis. In 80–90% of all cases, HCC is due to hepatitis B (HBV) and C (HCV) virus infections. Furthermore, because of diabetes and obesity, there is an increasing prevalence of non-alcoholic fatty liver disease (NAFLD), which is now the most common liver disorder in North America.3–6 The annual incidence of NAFLD-related HCC has increased by 9% per year from 2004 to 2009.6 Notable co-factors, such as alcohol, primary or secondary iron overload and aflatoxin contamination of stored food products are thought to play a synergistic role in promoting hepatocarcinogenesis, particularly in the context of HBV and non-alcoholic steatohepatitis.3,4 HCC is a complex disease due to its heterogeneity: from a clinical perspective in addition to the multiple aetiological risk factors, HCC typically has a prolonged asymptomatic phase early in the disease, and thus tends to present late with an aggressive phenotype that may not be amenable to currently available therapies. In terms of diagnosis and response to treatment, HCC displays several histopathological phenotypes, including, but not limited to, well and poorly differentiated tumours, and tumours exhibiting features of both hepatocellular and cholangiocarcinoma.3,7 At the molecular level, HCC is characterised by dysregulation of multiple genetic, epigenetic and signalling pathways that interact with the tumour microenvironment to facilitate tumour initiation, progression and metastasis. This review aims to concisely elucidate the current understanding of the molecular and cellular pathogenesis of HCC outlined in Table 1.

Cancer phenotype
Normal cells are originally embryonically and developmentally equivalent. However, they undergo a sequential process of cell fate determination, proliferation and differentiation. This process is dependent on extra-and intracellular interactions that are governed by various signalling pathways.4,7 Physiologically, these pathways are activated during early life but are dormant in adulthood. However, following inflammation or another insult, these pathways are re-activated, resulting in dysregulated cellular signalling, which accounts for the metamorphosis from normal to transformed malignant cells. Cancer cells, therefore, owing to genomic instability and/or mutations induced by cellular damage, have a distinct phenotype. These cells acquire the ability to: (i) autonomously proliferate (i.e. they are independent of external mitogenic signals); (ii) avoid both anti-growth and apoptotic signals, giving them a growth advantage; and (iii) deregulate certain cellular functions responsible for cellular growth and differentiation.9 Furthermore, cancer cells exploit signalling pathways to penetrate surrounding healthy tissue including the vascular epithelium, resulting in metastases to distant sites. Moreover, there is ample evidence that by their existence, cancer cells are able to suppress T-cell cytotoxicity and related immune mechanisms. This aggressive phenotype underpins the hallmark of carcinogenesis and explains malignant transformation. Therapies aimed at halting this autonomy need to be able to keep up with the many mechanisms involved; therefore, an understanding of the pathways is a pre-requisite.

Current concepts of hepatocarcinogenesis
Multi-step process
The currently accepted model of hepatocarcinogenesis is a multi-step process from tumour initiation to established malignancy. The evidence for step-wise progression of HCC is that normal hepatocytes are transformed to pre-neoplastic lesions, which occur in the form of dysplastic foci and nodules (DN) (<1 mm and >1 mm, respectively).10 With ongoing chronic inflammation, these early lesions progress to low- and high-grade dysplasia, both of which have the potential to progress to HCC (Fig. 1).11,12 The underlying
Other factors

Cellular factors

1. Genetic mutations
   - p53, TERT, others

2. Epigenetic
   - Changes to DNA, histones
   - Chromatin remodelling
   - MicroRNAs

3. Aberrant signalling
   - Tyrosine kinases
   - Wnt-β catenin
   - Notch
   - Hedgehog

4. Non-alcoholic fatty liver disease

5. Alcoholic liver disease

6. Haemachromatosis

7. Aflatoxin

Table 1. Summary of risk factors and molecular pathogenesis of hepatocellular carcinoma

<table>
<thead>
<tr>
<th>Main risk factors</th>
<th>Molecular factors</th>
<th>Cellular factors</th>
<th>Other factors</th>
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</table>
| 1. Cirrhosis      | 1. Genetic mutations  
                  |   • p53, TERT, others |
| 2. Hepatitis B virus | 2. Epigenetic   
                      |   • Changes to DNA, histones |
| 3. Hepatitis C virus | 3. Aberrant signalling 
                       |   • Tyrosine kinases |
| 4. Non-alcoholic fatty liver disease | | | |

Cancer stem cells

The long-held stochastic model of HCC pathogenesis states that damaged cells in tissue can randomly result in tumour initiation and/or growth. An attractive alternative theory is that within a tumour, a small population (<1%) of cells have phenotypic characteristics of adult progenitor stem cells, in that they have an inordinate capacity to autonomously proliferate and self-renew. As a result of a loss of regulation these cells accumulate, forming the bulk of the tumour (also called the cancer stem cell compartment) and are implicated in tumour initiation and maintenance. The expression of liver stem cell markers has been found in large numbers of human HCC, suggesting that human stem cells give rise to HCC. In fact CD133, one of the tumorigenic stem cell markers, was found in both HCC cell lines and primary tissues. Furthermore, the clinical relevance of these stem cells is that they have enhanced chemotherapy and radiotherapy resistance and are therefore typically associated with metastases and relapse. In order to more effectively attain better survival outcomes from currently available therapies including immunotherapy, further work into understanding the genetic and signalling pathways that regulate this cellular compartment is urgently required.

Molecular pathways involved

The requirement for carcinogenesis is a permissive milieu where genes and signalling pathways that regulate the fate of all cells, i.e. differentiation, proliferation and death, are altered. In this context, mutations of oncogenes or tumour suppressor genes in HCC become more important determinants (Fig. 2).

Genetic factors

Mutations of the telomerase promoter

Telomeres are protective nucleotide sequences capping the ends of chromosomes. These are particularly significant in the context of the liver in that the reparative capability of the telomerase enzyme affords hepatocytes their near-inexhaustible...
In this context, increased environmental influences on cancer can contribute to the development of HCC. There are several Wnt mutations of oncogenes, e.g. in the 3-dioxygenase; TERT = telomerase reverse transcriptase; lncRNAs = long non-coding RNAs.)

regenerative ability. However, when chronic inflammation occurs, the rate of telomere shortening is accelerated, which co-operates with inactivating mutations of telomerase to contribute to the development of cirrhosis. Under normal circumstances the telomerase enzyme is switched off to rid the body of senescent or abnormal cells. In HCC, however, mutations of the promoter region of the telomerase reverse transcriptase (TERT) allow malignant cells to evade apoptosis, resulting in an immortal phenotype. In HCC these are the most commonly described mutations, occurring in 29 - 60% of HCCs.

Mutations of the total protein 53 (TP53) pathway
TP53 has many anticancer functions including DNA repair, inhibition of G1/S cell cycle progression, and initiation of apoptosis by regulating the transcription of protective antioxidant genes and transactivating pro-oxidant genes. Inactivating mutations of TP53, are common in many cancers, not least in HCC, where they are present in 18 - 50% of cases. There are several variants of TP53 mutations in different cancers, which suggests a role for environmental influences on cancer phenotype. In HCC the most well-described TP53 mutation is a result of a transversion of G:C to T:A at codon 249, as a result of the synergism between aflatoxin B and HBV (particularly in endemic areas). The detection of TP53 mutant DNA in plasma is a biomarker of both AFB1 exposure and HCC risk.

Other mutations
There are many other genes involved in HCC that regulate proto-oncogene, tumour suppressor, signaling pathway, DNA-binding and other functions; these have been reviewed elsewhere.

Epigenetic factors
Epigenetics refers to heritable alterations in gene expression not due to changes of the genome itself that, under normal conditions, are used by the body to control processes such as X chromosome inactivation. Evidence exists, however, to suggest that changes in the epigenome are associated with HCC initiation and progression.

Epigenetic control is conferred by several mechanisms.

Modifications to DNA
The generally accepted mechanisms by which carcinogenesis occurs are global changes in the epigenome are associated with metastases and poor prognosis. Epigenetic gene silencing can also be mediated by a group of chromatin-modifying proteins known as polycomb repressive complexes (PRCs).

PRC1 and 2 are the chief epigenetic repressors involved in the maintenance of stem and adult cells and regulate repression by ubiquitination of group histone 2A lysine 119 (H2AK119), and tri-methylation of histone H3 lysine 27 (H3K27), respectively. Increased levels of EZH2, one of the components of the PRC2 complex, correlate with an aggressive HCC phenotype, associated with metastases and poor prognosis. Mechanistically, EZH2 silences Wnt antagonists, thereby activating Wnt-β-catenin signalling to promote cancer progression, whereas knockdown causes...
re-expression of tumour suppressor mRNAs, paying credence to its biological relevance.

Regulation by micro- and long non-coding RNAs

miRNAs (miRNAs) are 17 - 25-nucleotide-long non-coding RNA molecules that up- or de-regulate post-transcriptional gene expression by modifying the stability of or degrading mRNA. miRNAs/miR are important in the context of carcinogenesis because they regulate differentiation, development and oncogenesis. In addition to regulating various cellular processes, miRNAs are epigenetic modulators by targeting miRNAs of epigenetic regulators including DNA methyltransferase 3 alpha (DNMT3A), DNA methyltransferase 3 beta (DNMT3B), polycomb mRNAs, EZH2 (as shown above), BMI1 and HDAC4. Additional miRNAs that function as tumour suppressors include miR-26a, miR-26b, miR-125b, miR-140-5p, miR-217, miR-138, miR-148b, miR-325, miR-451. These are decreased in HCC, and are associated with a poor prognosis, therefore they may function as potential biomarkers for HCC. Reduced miR-26 expression correlates with shorter survival, but encouragingly, these patients are more likely to respond to interferon alpha therapy, making it an ideal candidate for predicting response to therapy.

Another group of non-coding RNAs (about 200 nucleotides in length) that regulate gene expression are the long non-coding RNAs (lncRNAs). Twenty percent of lncRNAs are associated with PRC2, through which they recruit and guide chromatin-modifying complexes to specific genomic regions to regulate gene expression. Other mechanisms of gene regulation by non-coding RNAs involve downregulation of tumour suppressor gene, activation of cell cycle function and chromatin reprogramming to promote metastases. These novel epigenetic regulators offer exciting opportunities for new therapies for HCC.

Signalling pathways

Several signalling pathways involved in all aspects of cell fate determination are exploited by cancerous cells to favour proliferation, growth, invasiveness and metastases. Although for clarity these will be discussed in separate sections based on their effect in tumour promotion, it is important to note that there is crosstalk between these pathways to mediate their effects. For example, Chung et al. show evidence of tripartite signal induction of the insulin/MAPK/ERK, Wnt and Notch pathways in a double transgenic mouse model of HBV/HBs protein to result in hepatocarcinogenesis.

Receptor tyrosine kinase pathways

Wnt-β-catenin

Wnt-β-catenin is one of the most studied and commonly implicated aberrant pathways in early HCC. Due to the multitude of ligands and receptors involved, it renders the effects of signalling through this pathway unpredictable, with some binding resulting in inhibition and others activation of signalling. Nonetheless, canonical Wnt signalling results in translocation of beta-catenin into the nucleus binding with TCF/LEF transcription factors coding for genes involved in cell proliferation angiogenesis, anti-apoptosis, and the formation of extracellular matrix (ECM), causing Wnt upregulation. Mechanisms of Wnt activation include somatic mutations of CTNNB1, AXIN1 and AXIN2, as well as inactivation of tumour suppressor adenosis polyposis coli (APC), which mimic pathway activation. Other mechanisms include epigenetic control of proteins of Wnt signalling.

Notch

The Notch pathway is a primitive and highly conserved pathway that is crucial in mammalian embryogenesis, cell fate determination, liver repair and regeneration. Its role in hepatic carcinogenesis is emerging; of the four Notch receptors, Notch 4 is well characterised as the most oncogenic, whereas Notch 1 may be either up- or downregulated. The function of Notch 3 appears minimal in HCC and that of Notch 4 is related to invasiveness and metastases rather than tumour initiation. Similar to the Wnt pathways, aberration in the pathway results in activation or inhibition of oncogenes and
tumour suppressor genes, respectively, and cross-talk with other pathways, the net effect of which may explain the heterogeneous phenotypic expression. Notch signalling is aberrantly upregulated in HCC compared with normal liver tissues.25,26

Hedgehog Activation of Hedgehog signalling was shown to be oncoenic for the first time when blocking of this pathway resulted in reduced proliferation, apoptosis and repressed C-myc and cyclin D expression, both in human HCC samples and liver cancer cell lines.27 Glioma-associated oncogene homolog-1 (Gli-1), a marker of Hedgehog pathway activation, is correlated to invasiveness and the risk of metastases in HCC. Inhibition of this pathway by small interfering RNA significantly suppressed adhesion, motility, migration and invasion of liver cancer cell lines and the expression and activities of both matrix metalloproteinases-2 and -9 (MMP-2 and MMP-9).19,28 Indeed, Hedgehog activation may be useful as a biomarker to delineate malignant from adjacent normal tissue and thus may be a useful target for local therapy,29 particularly as an inducer of apoptosis.30 Other mechanisms by which Hedgehog is oncogenic include the activation of MMP-9 through ERK.31 A key role of Hedgehog activation is that it is an inducer of radiation-induced liver fibrosis, which can be targeted with inhibitors to radiosensitive tumours prior to radiotherapy.32

Immunological tolerance The recent discovery and therapeutic potential of checkpoint inhibitors attests to the significant role of the immune system in the pathogenesis of HCC. The liver is an immunologically rich organ, elegantly poised to deal with gut-derived pathogens from the portal vein. More importantly however, is its adaptive ability to effect immune tolerance as a protective mechanism to avoid excessive liver injury. This is achieved through several key immunological mechanisms: liver sinusoidal endothelial cells have a high expression of programmed death ligand 1 (PD-1) and low expression of co-stimulatory CD80 and CD86,11,27 and downregulate MHC molecules and dendritic cell activation,33,34 thus curtailing their cytotoxic ability. In HCC specifically, there is immune exhaustion, typified by enhanced expression of co-inhibitory molecules PD-1, cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), lymphocyte-activation gene 3 (LAG3) and T cell immunoglobulin domain and mucin domain 3 (TIM-3),35 including decreased expression of effector cytokines, which limits cytotoxic effectiveness.36 Additionally, within the HCC tumour microenvironment, the immune response is directed towards an immunosuppressive phenotype with the release of anti-inflammatory cytokines, interleukin-10 and TGF-β.110 Other tumour evasion/escape strategies include the recruitment of immunosuppressive T-regulatory cells (T regs) and monocye-derived myeloid suppressor cells (MDSCs), and inhibitory indoleamine 2,3-dioxygenase (IDO), tryptophan 2,3-dioxygenase (TDO) and arginase-1 enzymes, which render immune cells deficient of tryptophan and arginine required for optimal functioning. The mechanisms by which malignant cells are able to thrive in this nutrient-deficient milieu are under investigation. However, a paper by Timosenko et al.37 describes the ability of HeLa cells to upregulate an amino acid transporter, solute carrier family 1 member 5 (SLC1A5), which imports tryptophan, whereas co-cultured T-cells were unable to do so, thus disabling their cytotoxic functioning.

Conclusion Despite decades of research into molecularly targeted therapies, including the recent advent of immunotherapy, the armamentarium against HCC is at best discouraging. None of these agents, including sorafenib, have translated into clinically meaningful improved patient survival. As such, HCC remains a deadly cancer. The understanding of all hepatocarcinogenic pathways is therefore critical to yield to new and effective therapies for HCC. Specifically, the exploration of epigenetic and immunological factors may more imminently result in faster progress towards alternative therapies. These efforts will require closer collaborations, not only between various medical disciplines, but also with basic/molecular biology scientists, immunologists, the pharmacological industry, and government bodies.

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