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Review

Tumor initiation and progression in hepatocellular carcinoma: risk factors, classification, and therapeutic targets

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Hepatocellular carcinoma (HCC) is a major health problem worldwide responsible for 500 000 deaths annually. A number of risk factors are associated with either the induction of the disease or its progression; these include infection with hepatitis B or C virus, alcohol consumption, non-alcoholic steatohepatitis and certain congenital disorders. In around 80% of the cases, HCC is associated with cirrhosis or advanced fibrosis and with inflammation and oxidative stress. In this review we focus firstly on the different risk factors for HCC and summarize the mechanisms by which each is considered to contribute to HCC. In the second part we look at the molecular processes involved in cancer progression. HCC development is recognized as a multistep process that normally develops over many years. Over this period several mutations accumulate in the cell and that stimulate malign transformation, growth, and metastatic behavior. Over the recent years it has become evident that not only the tumor cell itself but also the tumor microenviroment plays a major role in the development of a tumor. There is a direct link between the role of inflammation and cirrhosis with this microenviroment. Both *in vitro* and *in vivo* it has been shown that tumor formation and metastatic properties are linked to epithelial-mesenchymal transition (EMT), a process by which facillitates the tumor cell's attempts to migrate to a more favourable microenviroment. Several groups have analyzed the gene expression in HCC and its surrounding tissue by microarray and this has resulted in the molecular classification into a distinct number of classes. Here we also found a role for hypoxia induced gene expression leading to a clinically more aggressive gene expression in HCC. Molecular analysis also helped to identify important cellular pathways and possible therapeutic targets. The first molecule that in this way has shown clinical application for liver cancer is the multikinase inhibitor sorafenib, others are currently in different stages of clinical studies like the mTOR inhibitor everolimus.

Keywords: liver cancer; hypoxia; prognosis; targeted therapy; molecular classification

Hepatocellular carcinoma

Hepatocellular carcinoma (HCC) is a malignant tumour arising from the hepatocytes. HCC represents a major health problem. Worldwide, it ranks fifth among the solid tumours and it is the third cause of cancer-related mortality in males with ~500 000 deaths each year[1]. It is most prevalent in Asia (the annual incidence in China equals 100 per 100 000) and Africa. In Africa, HCC is mostly related to chronic hepatitis B virus (HBV) infection, in Asia to hepatitis B or C. The incidence in Europe and the USA is considerably lower with an annual incidence between 1–10 per 100 000, but it is rising mainly due to hepatitis C (HCV) virus infection with an expected fivefold increase peaking around 2015[2]. In more than 80% of cases, HCC is associated with cirrhosis or with advanced fibrosis[3, 4]. Cirrhosis is mostly due to chronic infection with the HBV or HCV, alcohol abuse, or metabolic disorders like hemochromatosis. More recently, non-alcoholic steatohepatitis (NASH) resulting from the insulin resistance syndrome is emerging as another important risk factor for cirrhosis and HCC[5]. The major risk factors for the development of HCC are summarized in Table 1.

Inflammation, chronic liver disease and cancer

Inflammation occurs in livers that are exposed to a wide variety of substances (hepatitis viruses, intermediates of alcohol metabolism, drugs *etc*)[7]. When the hepatic metabolism fails to convert drugs to non-reactive or non-immunogenic substances, the intermediates may damage hepatocytes or trigger Kupffer and other cells to release inflammatory cytokines and mediators. Viral proteins may also elicit an immune reaction, which can be equally detrimental for the intruder, the infected

Table 1. Risk factors for HCC. Known risk factors associated with hepato-cellular carcinoma (Adapted from Bosch *et al*, Gastroenterology 2004[6])

Major risk factors for HCC are:	
80%	Cirrhosis or advanced fibrosis, mostly due to
	- Hepatitis B virus infection
	- Hepatitis C virus infection
	- Alcohol
	- NASH
	- Congenital disorders such as Hemochromatosis, Wilson's disease, *etc.*
20%	In the absence of Cirrhosis or advanced fibrosis
	- Hepatitis B virus infection
	- Aflatoxin (mostly combined with HBV)
	- Some genetic disorders such as Tyrosinosis
	- Drug induced (*eg* anabolic steroids)

cell and the surrounding hepatocytes[8]. During the metabolisation of offending drugs or during immunological reactions, reactive oxygen species (ROS) are generated and this oxidative stress plays a central role in causing tissue damage, which will be discussed further[9].

A variety of cells cooperate in inflammatory reactions. Indeed, the liver lobule is formed by hepatocytes, cholangiocytes and non-parenchymal cells (Kupffer cells, sinusoidal endothelial cells and hepatic stellate cells (HSC). In addition, intrahepatic lymphocytes and liver-specific natural killer cells are present in the sinusoidal lumen and in the perisinusoidal space of Disse. Toxic substances as well as immunological responses per se can initiate a process of necrosis and stimulate inflammation through activation of Kupffer and HSC. The HSC will acquire a myofibroblastic phenotype in the various types of liver disease and this process can lead to liver fibrosis through the excess deposition of extracellular matrix. This deposition of extracellular matrix often results in reduction of the blood flow in parts of the liver[10, 11]. Fibrosis can develop into cirrhosis, which is responsible for significant morbidity and mortality and is one of the most important risk factors for the development of HCC. During cirrhosis irreversible, architectural changes occur with nodule formation, whereby hepatocytes lose their normal two-cell plate-like arrangement[12].

Cirrhosis is associated with the development of HCC, but the molecular basis for the cancer-promoting effect of cirrhosis remains far from clear. There is accumulating evidence in favor of a dysplasia-carcinoma sequence as has been identified in other types of cancer such as in colon carcinoma. It is hypothesized that the process of recurrent liver cell necrosis and regeneration with increased cell turn-over, renders liver cells more sensitive to the adverse effects of other mutagenic agents. Both genetic and epigenetic changes may occur, eventually leading to the subsequent formation of dysplastic foci, nodules and finally hepatocellular carcinoma[13, 14].

Certain mutagenic factors are associated with a specific genetic signature (such as aflatoxin), while other genetic changes across different etiologies are identified[15].

From an epidemiological and clinical point of view HBV, HCV and alcoholic cirrhosis are clearly the most important diseases for the development of HCC. From a pathophysiological perspective, oxidative stress represents an important pathway by which viruses or others risk factors exert their carcinogenic properties. Both hepatitis viruses and oxidative stress will be described in more detail in this review.

Hepatitis B virus

In adults, HBV infection mostly results in acute (rarely fulminant) hepatitis; in 10%, this infection will evolve to chronic hepatitis that can ultimately lead to the development of cirrhosis and eventually to hepatocellular carcinoma. In neonates or young children, the infection may run an asymptomatic chronic course, but this state of persistent HBV infection can also lead to the development of HCC[16]. Worldwide, more than 350 million individuals are infected with HBV and it is estimated that each year 1 million people die because of infection with this virus[17].

HBV belongs to the *Hepadnaviridae*, whose members can infect only certain species, being man or a specific animal such as chimpanzee, woodchuck, ground squirrel or duck. All *hepadnaviridae* display structural homology, infect primarily the liver and can cause hepatitis; chronic infection with (mammalian) hepadnaviridae can result in development of HCC[18].

HBV has a circular, partially double-stranded, DNA genome, consisting of four major, partially overlapping open-reading frames that encode for 4 completely different proteins: surface (S, PreS1, PreS2), Precore/C, DNA polymerase (P), and the X protein. The surface proteins (S, PreS1, and PreS2) are expressed on the surface of the infectious virion, or on "empty" particles present in the serum which are highly immunogenic[19]. The core protein has a structural function and may play an important role in the viral life cycle. The DNA polymerase gene product is a multifunctional protein that contains a primase or terminal protein, a spacer, DNA polymerase/reverse transcriptase and RNase. The P protein is the sole viral protein required for the correct priming of reverse transcription. The X gene encodes a basic protein that is a transcriptional trans-activator and can upregulate the activity of the HBV genome[20]. HBx also influences the expression of a number of host genes, especially genes involved in cell survival, cell cycle progression and invasion[21–23].

The link between HBV and HCC may be related to the inflammatory response to HBV infection and/or a direct role of the above mentioned gene products of HBV. The immune response to HBV-encoded antigens is responsible both for viral clearance and for disease pathogenesis. While the humoral antibody response to viral envelope antigens contributes to the clearance of circulating virus particles, the cellular immune response to the envelope, nucleocapsid, and polymerase antigens eliminates infected cells. The class I- and class II-restricted T cell responses to the virus are vigorous, polyclonal, and multispecific in acutely infected patients who successfully clear the virus, while the responses are weak and

more narrowly targeted in chronically infected patients[24].

The pathogenetic and antiviral potential of the cytotoxic T lymphocyte (CTL) response to HBV has been demonstrated by the induction of a severe necro-inflammatory liver disease following the adoptive transfer of HBsAg-specific CTL into HBV transgenic mice, and by the non-cytolytic suppression of viral gene expression and replication in the same animals by a post-transcriptional mechanism mediated by interferon gamma, tumour necrosis factor alpha, and interleukin 2[20]. The dominant cause of viral persistence during HBV infection is the development of a weak antiviral immune response to the viral antigens. While neonatal tolerance and juvenile partial tolerance seems to play an important role in viral persistence in patients infected at birth or before age 5 year, the basis for poor responsiveness in adult-onset infection is not well understood and requires further study. Some HLA types seem protective against chronic HBV infection[25, 26].

HBV can integrate integrate in the host genome where it can promote malignant transformation. HBV integration can cause microdeletions in the host DNA on positions that may involve cancer relevant genes such as MAPK and TERT[27]. HBV can also persist in the liver for many years in the so-called cccDNA form, the intermediate form required for viral reproduction[28]. Through this mechanism, there will be a continous immunological respons on newly produced viral antigens in these chronically infected patients that can contribute to cellular damage and malignant transformation (see also oxidative stress). Data related to the influence of different hepatitis B virus genotypes and the emergence of selective variants as biomarkers of HCC development still remain controversial. Recent studies on occult HBV infection, as defined by serologically undetectable hepatitis B surface antigen (HBsAg-) despite the presence of circulating HBV DNA, suggest that the occult viral strains, maintaining the transcriptional activity and the pro-oncogenetic assets of the clear HBV infection (HBsAg+), may harbour a potential risk for liver cancer development[29].

Concerning the gene products of HBV, there is a central role for the HBV X gene. HBx is a transcriptional factor that regulates gene expression of several oncogenes[30, 31] and its carcinogenic potential was demonstrated in transgenic mice who developed HCC in 90% of the cases[32]. HBx has been shown to directly impair insulin signaling which suggest some common mechanism between HBV induced HCC and in fatty liver disease/NASH/NAFLD[33].

Also, the preS2/S protein of HBV is linked to HCC development via activation of hTERT and so to HCC [34].

Chronic liver cell injury and the inflammatory and regenerative responses are believed to create the mutagenic and mitogenic stimuli leading to HCC.

Hepatitis C virus

Hepatitis C virus infection (HCV) is currently the most common cause of chronic liver disease in the USA, Australia, Japan and Western Europe. As the infection is usually acquired in adult life through the parenteral route and because progression of chronic hepatitis over cirrhosis to cancer can take several decades, most patients are of late middle or old age when they are diagnosed with HCC[35]. However, HCV is associated with a higher risk to induce chronic infection (60%–80% *vs* 10% in HBV) and therefore with HCC development as well compared to HBV[36].

The rate of fibrosis progression is highly variable, and is influenced by the amount of alcohol consumption, age at the time of HCV infection, degree of inflammation and co-infection with HIV or HBV. HCC occurs in approximately 2% of cirrhotic patients per year.

The HCV is a single-stranded positive-sense RNA virus of 9400 bp. The HCV genome contains a single large open-reading frame encoding a polyprotein precursor of 3010–3033 amino acids, depending on the viral isolate. HCV structural genes (core and envelope) are encoded at the 5' region of the genome. Non-structural genes occupy the remaining two-thirds of the 3' region of the viral genome. Non-coding regions of the virus are crucial to viral replication and are found at both ends of the genome[37].

The life cycle of HCV is confined to the cytoplasm of the host cell. The viral genome does not integrate into the host cell genome and does not express a reverse transcriptase as HBV does. Therefore its role in carcinogenesis has generally been assumed to be mediated by the chronic necro-inflammatory process. Negative replicative and positive genomic strands of the virus are present in both liver and tumour tissue; this indicates that HCV not only infects hepatocytes but also replicates in them during, and possibly even following malignant transformation[37]. In addition to necro-inflammatory processes, direct effects of viral proteins on the host cell can also play a role in the malignant transformation of the hepatocytes to HCC.

The HCV core protein seems to be involved in overcoming apoptosis since it interferes with for example TNFα signaling[38]. HCV core protein in particular also can induce insulin resistance and this might further trigger development of HCC[39, 40]. A link with insulin has also been found for HBV (see above). Liver cancer-derived hepatitis C virus core proteins shift TGF-beta responses from tumor suppression to epithelial-mesenchymal transition which is discussed below[41].

Mutations in the hepatitis C virus core gene are associated with advanced liver disease and hepatocellular carcinoma[42]. And there has been found an association between mutations in the core region of hepatitis C virus genotype 1 and hepatocellular carcinoma development[43].

Non-structural 5A protein of hepatitis C virus induces a range of liver pathology in transgenic mice[44] and Hepatitis C virus NS5A protein is shown to interacts with beta-catenin and stimulates its transcriptional activity in a phosphoinositide-3 kinase-dependent fashion[45].

Hepatocellular carcinoma displays distinct DNA methylation signatures[46, 47], including genes inhibitory to cancer-related signaling such as RAS/RAF/ERK and Wnt/beta-catenin pathways. Also, in a group of patients the methylation of dual specificity phosphatase 4 (DUSP4), cytochrome P450, family 24, subfamily A, polypeptide 1 (CYP24A1), and

natriuretic peptide receptor A (NPR1) correlated significantly with recurrence-free survival. Deng *et al* concluded that DNA methylation might play an important role in HCV-related HCC by silencing cancer-related pathway inhibitors[47].

Alcohol, steatohepatitis, and related fatty liver diseases

Fatty liver disease is a heterogeneous condition characterized by fatty degenerative changes of hepatocytes, induced by alcohol, insulin resistance or metabolic diseases. In the clinic fatty liver disease is commonly divided into two conditions: alcoholic and non-alcoholic fatty liver disease (NAFLD)[48].

The histology of fatty liver diseases extends from steatosis to steatohepatitis, fibrosis and cirrhosis. Alcoholic or non-alcoholic steatohepatitis (ASH or NASH) is characterized by the accumulation of fat droplets in the cytoplasm of hepatocytes and the additional presence of various degrees of lobular inflammation, hepatocyte-ballooning, -apoptosis, and -necrosis, and fibrosis. In obese persons, the prevalence of NAFLD reaches as much as 75% to 90%[49].

The principal risk factors for NAFLD are visceral obesity, type 2 diabetes mellitus, hypertension and hyperlipidemia.

Several mechanisms have been proposed to explain carcinogenesis in (N)ASH[50–55]. But in both cases inflammation and the generation of reactive oxygen species play a pivotal role. Chronic alcohol abuse leads to multiple biochemical changes that could indirectly initiate or potentiate liver cancer. Alcohol is metabolized to acetaldehyde by alcohol dehydrogenase and by cytochrome P450 2E1 (CYP2E1). Both steps lead to the production of NADH, which can lead to oxidative stress and gluthatione depletion. This will be explained in more detail later. Furthermore, acetaldehyde is considered as a carcinogen in animal studies. In addition, animal studies have shown that the production of S-adenosylmethionine (SAM), the methyl donor for DNA methylation reactions, is decreased in alcohol-induced cirrhosis. Because DNA methylation is an important regulator of gene expression, epigenetic abnormalities in DNA methylation in alcohol-induced cirrhosis could also contribute to changes in gene expression and the development of HCC[56].

Oxidative stress and HCC

Oxidative stress is caused by an imbalance between on one hand, the production of ROS and reactive nitrogen species (RNS) and on the other hand, the ability to detoxify these reactive intermediates or to easily repair the resulting damage. All forms of life maintain a reducing environment within their cells. The cellular redox environment is preserved by enzymes that can maintain this reduced state through a constant input of metabolic energy. Disturbances in the normal redox state can have toxic effects by overproduction of peroxides and free radicals.

In humans, oxidative stress is thought to be involved in many diseases, such as atherosclerosis, liver disease, Parkinson's disease, Alzheimer's disease and ageing[57, 58]. However, reactive oxygen species can also be beneficial, as they are used by the immune system as a way to attack and kill pathogens and by the cell as signaling molecules.

In case of liver disease, ROS and RNS seem to play a crucial role in the pathogenesis and the progression of HCC irrespective of the etiology. ROS and RNS are involved in the transcription and activation of a large series of cytokines and growth factors, which in turn, can contribute to further production of ROS and RNS. Hepatocyte mitochondria and cytochrome P450 enzymes, endotoxin-activated macrophages (Kupffer cells) and neutrophiles represent the main sources of free radicals. These free radicals will alter the redox state, and the cellular tissue damage may be potentiated by an associated decrease of antioxidant and energetic reserves. These radicals have a short half-life but they can react with many classes of molecules or compounds (proteins, membranes, DNA and RNA)[59].

To counteract the effect of oxidative stress, cells have developed two important defense mechanisms: redox active sulfhydryl systems [glutathione (GSH) and thioredoxine (TRX) and enzymatic systems (including superoxide dismutase, catalase and glutathione peroxidase)][60]. Glutathione is the most abundant non-protein sulfhydryl-containing compound in the cell and constitutes the major thiol buffer.

There is considerable evidence that redox signaling mechanisms function in cell regulation and growth control. Not only is cellular GSH involved in proliferation, changes in the cellular GSH/Glutathione disulfide (GSSG) redox state (oxidative stress) have been implicated in cell cycle responses such as differentiation and apoptosis[61]. The redox status of the cells can be calculated by the Nernst equation for the couple GSSG/2GSH. Another marker for oxidative stress is malondialdehyde (MDA) that is formed during lipid peroxidation and was shown to accumulate in serum of chronic hepatitis patients[62]. An imbalance in the redox homeostasis results in an increased flux of reactive oxygen species[63]. Under such pro-oxidant conditions, highly reactive radicals can damage DNA, RNA, proteins and lipid components, and this may lead to mutations or apoptosis[61, 64].

Eukaryotic cells possess evolutionary conserved mechanisms to accurately and efficiently repair the majority of DNA damage, thereby ensuring genomic integrity. Important repair pathways include base excision repair, nucleotide excision repair, mismatch repair, non-homologous end joining and homologous recombination. Defects or reduction in DNA repair processes generally result in susceptibility to cancer. Both hepatitis B virus and oxidative stress itself have been shown to interfere with DNA repair and may increase de novo HBV DNA integration[65, 66].

Hepatocarcinogenesis and tumormicroenviroment

Hepatocarcinogenesis is a multistep process in which a number of mutational genetic alterations accumulate in a cell (Figure 1). It involves the transition of a normal cell via the so called initiated cell to a preneoplastic lesion that develops into malignant tumour and to clinical liver cancer[67].

Hepatocytes in normal adult liver hardly ever proliferate and have a life span of over a year. After extensive liver damage or in situations where hepatocyte regeneration after dam-
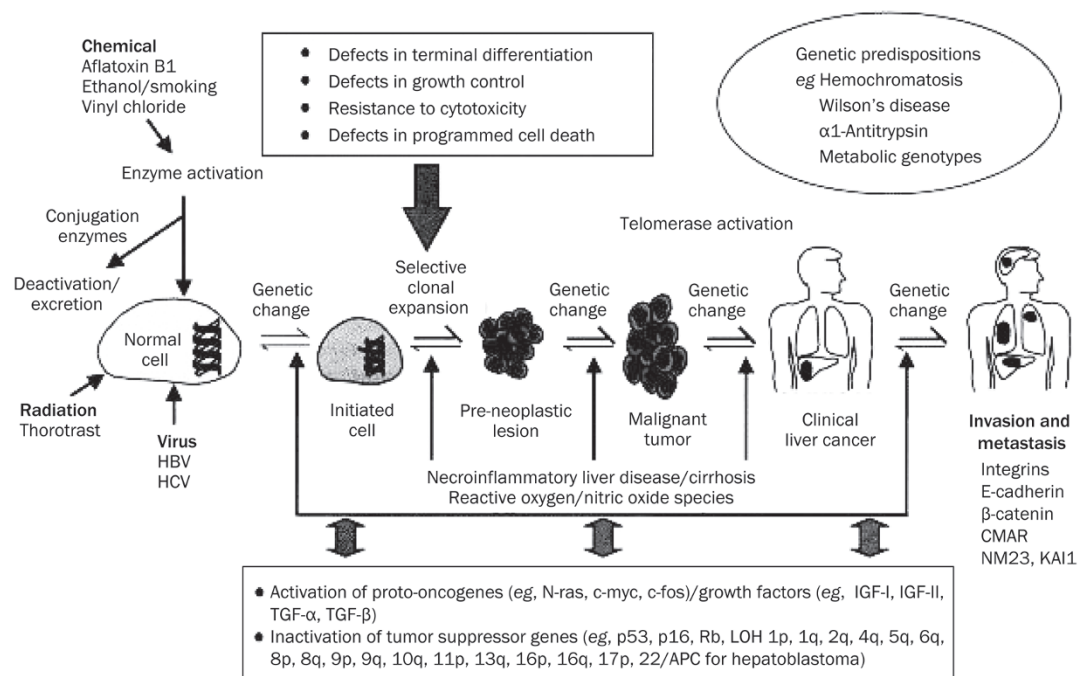
Figure 1. Schematic representation of the multistep process of hepatocarcinogenesis (from Wang *et al*, Toxicology 2002[68] with permission).

age is compromised, proliferation of a (facultative) stem cell compartment is observed, consisting of the hepatic progenitor cells (HPC) located in the canals of Hering near the smallest branches of the biliary tree. These cells are bipotential and capable of differentiating into either hepatocytes or cholangiocytes. These HPC's are small cells with a large nucleus and a narrow rim of cytoplasm, in animals also called oval cells. The degree of this progenitor cell activation is positively correlated with the inflammatory activity and the stage of the disease. Recently, it has been shown that in the cirrhotic stage of most chronic liver diseases, the hepatocytes become senescent owing to telomere shortening. Some senescent cells acquire additional mutations and an associated gain in replicative capacity this makes it even more plausible that at least part of the hepatocellular carcinomas originate from a progenitor cell.

Using immunohistochemistry (OV-6 and GST-P) and RNA-FISH (HGF, EGF, CK19, and H19) identified isolated stem cells scattered in the liver parenchyma of cirrhotic livers and within hepatocellular carcinomas (HCCs). Precirrhotic ASH or NASH all stained negative for these stem cells. In HCCs, 13 out of 15 had stem cells located within the tumor (78%). In cirrhotic livers, 12 out of 28 (37%) had liver parenchymal stem cells present[69].

Several studies suggest that HCC with stem cell features have a poor prognosis. Lee *et al* showed significant survival differences between HCC patients with a hepatocyte-like phenotype and a hepatoblast-like phenotype[70, 71]. Other studies suggest that HCC positive for Keratin 19 (KRT19), which is a progenitor cell marker, have a higher rate of recurrence after resection or transplantation[72–74]. Other fetal markers such as AFP and epithelial cell adhesion molecule (EpCAM) are of prognostic value as well, since EpCAM+/AFP+HCC is associated with poor survival[75].

Supported by a growing number of scientific reports it has become clear over recent years that also the tumor enviroment plays an important role in tumorigenesis and tumor progression[76]. Cancer cells are not as autonomous as once thought, they depend on angiogenesis, inflammatory cells and fibroblasts[77, 78]. Obvious, in the cirrhotic liver there is an abundancy of fibroblasts and there is emerging evidence that some are cancer associated fibroblasts (CAFs)[79]. In HCC, the progression of malignant hepatocytes frequently depends on transforming growth factor (TGF)-beta provided by the stromal cells (fibroblasts, macrophages *etc*). This TGF-beta is one of the factors that can induce an Epithelial-to-Mesenchymal Transition (EMT)[80]. For different types of tumors it has been demonstrated that an EMT-switch is associated with a worse prognosis as seen in esophageal squamous cell carcinoom[81], gastric cancer cells[82] bladder cancer[83] non-small cell lung cancer[84] and pancreas ductal adenomacarcinoma[85]. For HCC, this correlation has also been found with an independent effect of Twist and Snail in promoting metastasis of hepatocellular carcinoma[86–89].

Characteristics for EMT are a shift in gene expression with an upregulation of Twist[85,90], Snail[91], VE-cadherin[92], Vimentin[93], downregulation of E-cadherin[94], hepatocyte transcriptional factor HNF4alpha and changes in the cytoskeleton. Important molecular pathway involved in EMT are Wnt/beta-catenin signaling[95, 96] and through growth factor signalling like PDGF[97].

In EMT there is a de-stabilization of adherent junctions[98] and the cells develop more invasive properties[93].

In mouse models, both *in vitro* and *in vivo*, epithelial-to-mesenchymal transition has been shown to promote invasion and metastasis[99, 100].

Both hepatitis B virus[101] and hepatitis C virus[102] are fac-

tors that contribute to EMT in HCC tumor progression. The tumormicroenviroments is also influenced by hypoxia through Hif1a[103] and hypoxia might stimulate EMT[104].

Kim *et al* has shown that epithelial-to-mesenchymal transition gene signature can predict clinical outcome of hepatocellular carcinoma. They used RT-PCR to determine expression of EMT-related genes and could predict survival of patients using only four genes (E-cadherin (CDH1), inhibitor of DNA binding 2 (ID2), matrix metaloproteinase 9 (MMP9), and transcription factor 3 (TCF3)[105]. Both this gene signature and the hypoxia signature (discussed below) are candidates to be included in a more comprehensive molecular classification of HCC and could supply us with valuable therapeutic targets.

Molecular classification

Several studies have tried to classify HCC based on their gene expression or on their chromosomal alteration. Obtaining a molecular classification of hepatocellular carcinoma, however, remains a striking challenge because of the overwhelming genomic complexity of HCC[106]. Hitherto, there are three large studies published that classify HCC based on gene expression. Two of them could identify subgroups with common affected pathways such as beta-catenin and proliferation signals[107, 108] and one group[70, 109] was able to correlate the subgroups with survival. Other studies tried to find common affected genes that are associated with prognostic factors such as vascular invasion and metastasis, but the overlap between these studies is very poor. Two groups studied the surrounding liver tissue, instead of the tumor tissue itself. Based on these findings late recurrence can be predicted in cirrhotic patients, since these recurrences are probably "second primary tumors in an at-risk liver". Table 2 summarizes some of the most important studies regarding the molecular classifications of hepatocellular carcinoma.

These studies led to the transcriptome classification as developed by Zucman-Rossi and Boyault (Figure 2).

A subgroup of patients have activation of pathways related to cell proliferation (IGF signaling, RAS/MAPK, and mTOR signaling), and differentiation (Wnt-β-catenin and Hedgehog). Overall we can classify as follows: 1) a proliferation subgroup with Akt/mTOR activation, 2) the closely related DNA-repair/Cell-cycle subgroup, 3) the Beta-catenin subgroup, 4) the immune related subgroup, 5) the MAPK/cjun/c-Myc related genes and Jak/STAT related genes and 6) the metastasis related genes group. The prognostic implication of this clustering based on pathways remains unclear.

Hypoxia and tumor progression

The role of hypoxia in initiation and progression of HCC is only partly understood. Hypoxia is involved in neo-angiogenesis and the formation of new blood vessels, induction of gene expression through the transcription factor Hif1α that regulates more than 50 targets genes and also hypoxia stimulates EMT in HCC[120] involving PI3 kinase/AKT signaling[121–123].

From our own recent studies it has come forward that hypoxia has an additional role. Central in our study was the working hypothesis that chronic exposure to hypoxia leads to an adaptive gene expression profile which influences the aggressive behavior of the tumor cells. To test our hypothesis independently of patient selection and variability, we decided to start from cell culture. We determined the gene expression pattern for human HepG2 liver cells under chronic hypoxia by microarray. Differentially expressed genes were selected and their clinical value was assessed. In our hypothesis-driven analysis we included available independent microarray studies of patients with HCC in one single analysis. Three microarray studies were used as training sets to determine a minimal prognostic gene set and one additional study was used for validation. Using computational methods we identified 7 genes, out of 3592 differentially expressed under chronic hypoxia, that showed correlation with poor prognostic indicators in all training sets (272 patients) and this was validated
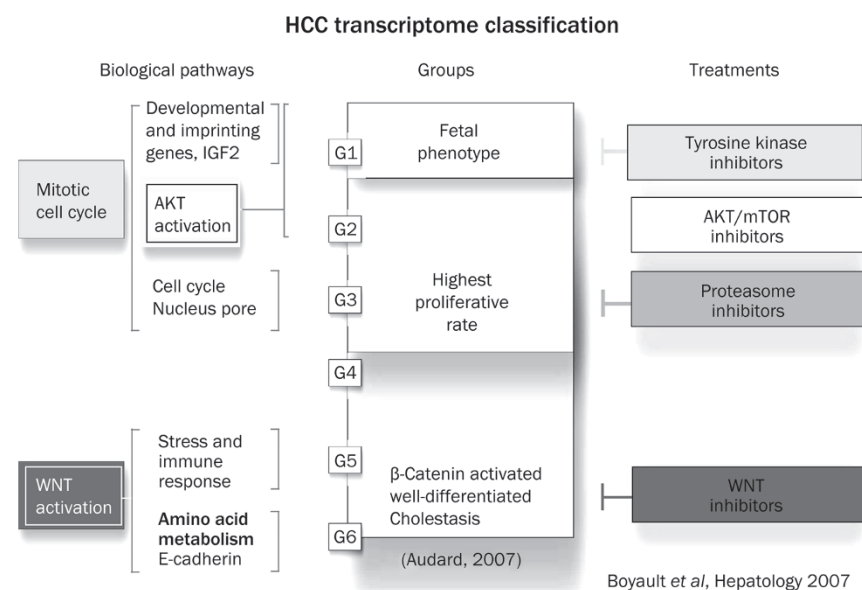
Figure 2. Transcriptome classification of HCC. HCC were divided into six groups with their main biological and molecular features and their implications for the selection of targeted therapies (after Zucman-Rossi *et al*[107] with modifications[119]).

Table 2. Overview of different studies on HCC in which microarray technique were used.

Study	Study aim	HCC type *n* (HCC)	Subgroups

Boyault/Laurent-puig [107]	Classification	All
		n=120
			2 groups (with each 3 subgroups):
			G1–3: high proliferation, chromosomal instability, activation of IGF or Akt/mTOR signaling
			  - G2–3: frequent p53 mutations
			G4–6: Chromosomal stability
			  - G5–6: activation of Wnt signaling, high prevalence of CTNNB1 mutations

Chiang [108]	Classification	HCV induced *n*=103	5 subgroups (no association with prognosis) - CTNNB - Proliferation (IGF/mTOR icm high AFP) - IFN related - Polysomy chromosome 7 (c-Met) - Unannotated
Wurmbach [110]	Classification	HCV induced *n*=35	Dyplasia Early HCC (Pathways affected — up: Wnt, down: Jak/stat, insulin, TGF-β, Toll-like receptor Advanced HCC (cell cycle — and DNA repair genes)
Breuhahn [111]	Classification	All *n*=34	2 subgroups A) Upregulation of IFN-related genes (better prognosis) B) Downregulation of IFN-related genes and apoptosis - B1 Overexpression IGF2 - B2 Downregulation IGF2, FAF1, TNIP1
Lee [70,109]	Survival	All *n*=139	3 subgroups A: Proliferation genes, high AFP; poor prognosis B: Opposite of A or smaller changes; good prognosis HB: fetal features, genes associated with invasion; very poor prognosis
Iizuka [112]	Recurrence	All *n*=60	Genes associated with early (<1 yr) recurrence after 'curative' resection
Woo [113]	Recurrence	HBV *n*=65	Genes associated with high risk of recurrence
Ye [114]	Metastasis	HBV induced *n*=67	2 subgroups - metastatic - non-metastatic
Chen [115]	Vascular invasion	All *n*=102	61 genes were associated with presence of vascular invasion. Of which MMP14 and ADAMTS1 are the most important
Okabe [116]	Vascular invasion	HBV+HCV *n*=20	Genes involved in cell motility were altered
Surrounding liver tissue Hoshida [117]	Survival/Recurrence	All *n*=82 (validation in *n*=225)	2 subgroups I – Good prognosis — Genes associated with normal liver function (ADH5/6/9A1, AKR1A1/D1, CYP2B6) II – Poor prognosis/High recurrence risk — Associated with inflammation (IFN signaling, NF-κB, TNF-α, IL-6)
Budhu [118]	Metastasis	90% HBV	2 subgroups - metastatic (increase in Th2 cytokines, decrease in Th1 cytokines) - non-metastatic

in a 4th dataset (91 patients). Retrospectively the 7-gene set is associated with poor survival (HR 1.39, $P=0.007$) and early recurrence (HR 2.92, $P=0.007$) in 135 patients. Moreover, using a hypoxia score based on this 7-gene set we found that patients with a score >0.35 had a median survival of 307 days, whereas patients with a score ≤0.35 had a median survival of 1602 days ($P=0.005$)[124].

These findings may have prognostic implications for HCC patients and therefore could be incorporated in the molecular classification of HCC. Furthermore, we suggest that gene expression under chronic hypoxia might lead to new therapeutic targets and the hypothesis-driven experimental methods we used can be applied to other malignancies as well.

Therapeutic targets
Dysregulation of pleiotropic growth factors, receptors and their downstream signaling pathway components represent a central pro-tumourigenic principle in human hepatocarcinogenesis.

Especially the Insulin-like Growth Factor/IGF-1 receptor (IGF/IGF-1R), Hepatocyte Growth Factor (HGF/MET), Wingless (Wnt/beta-catenin/FZD), Transforming Growth Factor alpha/Epidermal Growth Factor receptor (TGFalpha/EGFR) and Transforming Growth Factor beta (TGFbeta/TGFbetaR) pathways contribute to proliferation, anti-apoptosis and invasive behavior of tumour cells. Resultant functional effects are modulated by multiple cross-talks between the different signaling pathways and additional tumour-relevant factors, such as cyclooxygenase-2 and p53. With the identification of relevant pathways, specific therapeutic strategies can be developed. Some of these malignant signaling pathways have been identified and have been studied to develop new therapies[106, 125, 126].

Treatment
Treatment of patients with HCC is based on staging, which includes assessment of tumour extent, liver function, portal pressure and clinical performance status. The widely used Barcelona-Clinic-Liver-Cancer (BCLC) staging system links staging of HCC in cirrhosis with treatment modalities[127, 128]. The system identifies those patients with early HCC (stage 0 and A) who may benefit form curative therapies (resection, radiofrequency ablation, liver transplantation), those at intermediate or advanced stage (stage B, C) who may benefit from palliative treatments (such as transarterial chemo- or radioembolisation), and the patients with a very poor life expectancy (stage D) where best supportive care is the only option[129].

Until recently, patients with advanced hepatocellular carcinoma (BCLC-stage C) could only be offered best supportive care or systemic chemotherapy with an unfavourable risk-benefit ratio. The advent of targeted drugs has offered hope to our patients. Sorafenib is a multikinase inhibitor targeting the Raf serine/threonine kinases and the VEGFR1-3, PDGFR-β, c-Kit, Flt3, and p38 tyrosine kinases, that block the VEGF and PDGF-dependent angiogenesis. The drug is now considered the standard of care in patients with advanced hepatocel-

lular carcinoma and preserved liver function, based upon two phase III trials that showed an improved overall survival and progression free survival compared to placebo. Median survival increased from 7.9 to 10.7 months (hazard ratio 0.69, 95% CI 0.55–0.87)[130, 131]. Most drug-related and manageable adverse events include fatigue, anorexia, diarrhea and hand-foot skin reaction. Unfortunately, all patients with advanced HCC still die from the disease and there is an unmet need for other drugs, either as a single agent or in combination with sorafenib. A phase III clinical trial with sunitinib, a drug with a similar mode of action as sorafanib, has recently been stopped because of higher toxicity and no superior efficacy compared to sorafenib. Other clinical trials explore the safety and efficacy of everolimus that targets mTOR, which is a central regulator of cell growth and angiogenesis.

So far, the molecular classification of HCC has not yet found its place in the staging systems and selection of therapy. However, the active search and analysis for biomarkers in all current phase III studies may rapidly change the field.

Conclusions and perspectives
As pointed out in this review, HCC is a complex disease which can have different etiological origin. The disease is further complicated because it usually requires multiple hits (mutations), develops over many years and its clinical diagnosis is often at an advanced stage. The driving molecular mechanisms are under study and several groups have worked at the development of molecular classification systems, which provide road maps for the identification of therapeutic targets. Our studies on the role of hypoxia as a factor inducing a more aggressive gene expression profile, yielded an additional pathway attractive for therapeutic intervention. So far, treatment has been based on clinical staging systems. With the increasing knowledge of the biology of HCC a rational, individualized approach will become possible for the treatment of patients. In the future the models for molecular classification will further merge with the current staging and treatment algorithms. The discovery of sorafenib as an active and safe drug for patients with advanced HCC serves as a proof of principle clearly boosted the field and offers hope for the patients and the oncologic community.

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