# 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] These figures attest to the dismal post-diagnosis outlook (3.4 months median survival) with or without screening.[2] 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] The annual incidence of NAFLD-related HCC has increased by 9% per year from 2004 to 2009.[4] 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.[5,6] 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.[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.[8] 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

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

| Main risk factors | Molecular factors | Cellular factors | Other factors |
|---|---|---|---|
| 1. Cirrhosis | 1. Genetic mutations<br>• *p53*, TERT, others | 1. Cancer stem cells | 1. Checkpoint inhibitors |
| 2. Hepatitis B virus | 2. Epigenetic<br>• Changes to DNA, histones<br>• Chromatin remodelling<br>• MicroRNAs | 2. Immune cells<br>• Tregs<br>• MDSCs | 2. Immunosuppressive enzymes<br>• IDO<br>• Arginase |
| 3. Hepatitis C virus | 3. Aberrant signalling<br>• Tyrosine kinases<br>• *Wnt*-β catenin<br>• Notch<br>• Hedgehog | | |
| 4. Non-alcoholic fatty liver disease | | | |
| 5. Alcoholic liver disease | | | |
| 6. Haemachromatosis | | | |
| 7. Aflatoxin | | | |

p53 = total protein 53; TERT = telomerase reverse transcriptase; Tregs = T-regulatory cells; MDSCs = myeloid-derived suppressor cells; IDO = indoleamine 2,3-dioxygenase XX; *Wnt*-β = Wnt-Beta catenin.

mechanism of sequential progression is incompletely understood but is thought to be due to progressive hepatocyte dedifferentiation due to impaired liver-specific gene expression and the alteration of numerous signalling pathways, leading to dysregulated cell proliferation and resistance to apoptosis.[13] In patients with HCC gene expression, patterns of cell proliferation markers and anti-apoptotic genes were significantly higher in the group of patients with poorer prognosis, lending credence to their significance in HCC pathogenesis.[14,15]

## 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.[16,17] 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.[18] In fact CD133, one of the tumorigenic stem cell markers, was found in both HCC cell lines and primary tissues.[19,20] 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.[20,21] 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.

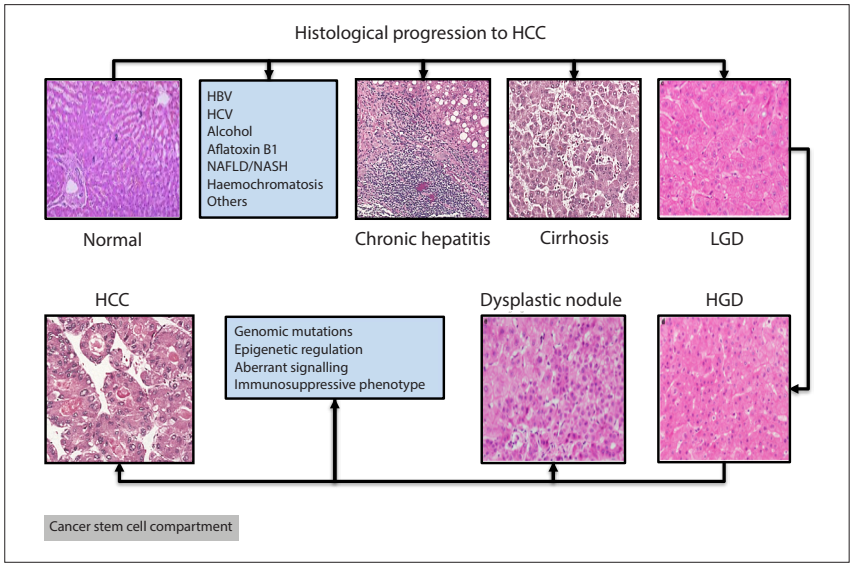


*Fig. 1. Progression to hepatocellular carcinoma (HCC) starts with a chronic insult to the liver resulting in chronic hepatitis and ultimately cirrhosis, the chief risk factor for HCC. In a stepwise fashion, lesions progress from low-grade dysplasia (LGD), to high-grade dysplasia (HGD), dysplastic nodules and finally HCC. HCC may be well or poorly differentiated (not shown). (NAFLD/NASH = non-alcoholic fatty liver disease/non-alcoholic steatohepatitis.)*

# 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

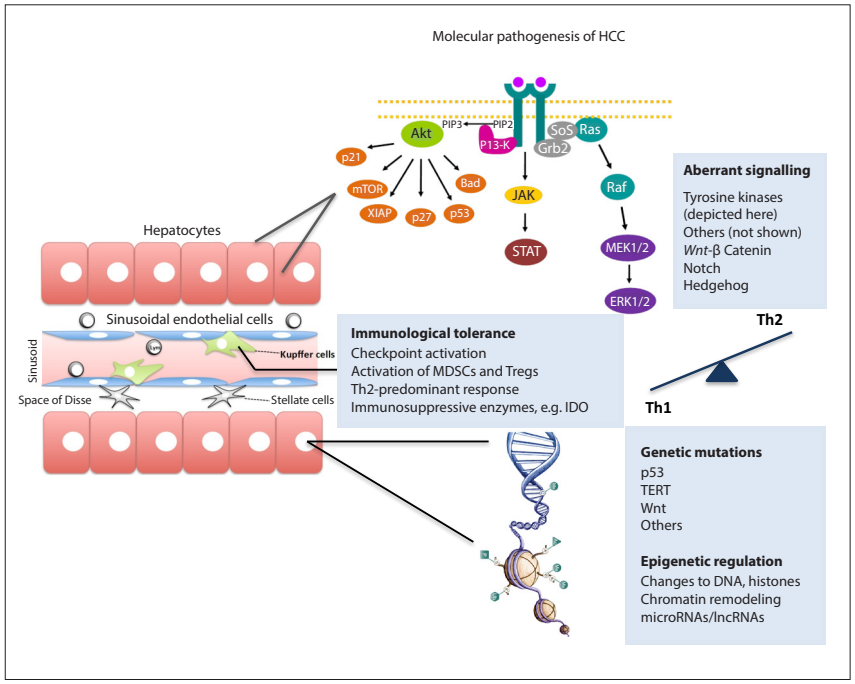


*Fig. 2. Molecular pathogenesis of HCC is an interaction between aberrant signalling, key genetic mutations, epigenetic control of gene expression and induction of immunological tolerance. (MDSCs = myeloid derived suppressor cells; Tregs = T-regulatory cells; IDO = indoleamine 2,3-dioxygenase; TERT = 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.[22,23] 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.[24,25]

### 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.[26] Inactivating mutations of TP53, are common in many cancers, not least in HCC, where they are present in 18 - 50% of cases.[26,27] There are several variants of TP53 mutations in different cancers,[28] 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).[27,29,30] The detection of TP53 mutant DNA in plasma is a biomarker of both AFB(1) 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.[7,31]

## 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.[32,33] Evidence exists, however, to suggest that changes in the epigenome are associated with HCC initiation and progression.[34] Epigenetic control is conferred by several mechanisms.

### Modifications to DNA

The generally accepted mechanisms by which carcinogenesis occurs are global hypomethylation resulting in activating mutations of oncogenes, e.g. in the *Wnt* pathway.[35] In HCC, however, the hypermethylation of promoter regions of tumour suppressor genes is more typical and results in their silencing[36] by either inhibiting the interaction of transcription factors with their promoter, or binding of methyl-CpG binding domain proteins, to methylated DNA.[37,38] Genome-wide methylation profiling studies have identified multiple hypermethylated gene promoters including adenosis polyposis coli (APC) and others in HCC tumours compared with surrounding non-tumour tissue.[39-44] This is clinically relevant because, for instance, low levels of sphingomyelin phosphodiesterase 3 (SMPD3), a potent tumour suppressor, were three times more likely to be associated with early recurrence of HCC after curative surgery in an independent patient cohort.[45,46] In this context, therefore, methylation profiling holds promise in terms of predicting patients who are more likely to progress to HCC.

### Modifications to histones

Post-translational modifications resulting in an open or closed configuration of histone proteins, which affects their accessibility, have a significant effect on the 'on' or 'off' state of gene expression.[47] While acetylation by histone acetyltransferases (HATs) causes activating transcription of genes,[48] histone deacetylases (HDACs) result in tight coiling of DNA around the histones, leading to transcriptional repression.[49] By contrast, methylation confers a dual role of activation or repression, which is context-specific. For example, tri-methylation of lysine 4 (K4) and 36 on histone 3 (H3K4me3 and H3K36Me3) are transcriptionally active start sites (TSS) of active genes.[50-52] Histone H3 lysine 4 (H3K27me3) is significantly elevated in patients with HCC, and this correlates with a poor prognosis (3.5-fold increased risk of death) as a result of aggressive tumour features, including vascular invasion, large tumour size and poor differentiation.[53,54]

### Chromatin remodelling

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.[55] Increased levels of EZH2, one of the components of the PRC2 complex, correlate with an aggressive HCC phenotype, associated with metastases and poor prognosis.[54,56] Mechanistically, EZH2 silences *Wnt* antagonists, thereby activating *Wnt*-β catenin signalling to promote cancer progression,[57] whereas knockdown causes

re-expression of tumour suppressor mRNAs,[58] paying credence to its biological relevance.

**Regulation by micro- and long non-coding RNAs**

MicroRNAs (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.[59,60] miRNAs/miR are important in the context of carcinogenesis because they regulate differentiation, development and oncogenesis.[61] In addition to regulating various cellular processes, miRNAs are epigenetic modulators by targeting mRNAs of epigenetic regulators including DNA methyltransferase 3 alpha (DNMT3A), DNA methyltransferase 3 Beta (DNMT3B), polycomb mRNAs, EZH2 (as shown above), BMI1 and HDAC4.[62,63] miR-122 is most abundant in the liver and is frequently downregulated in HCC, which suggests its role as a tumour suppressor.[64,65] 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.[36,63] 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.[66]

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.[67] 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.[68] 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.*[69] show evidence of tripartite signal induction of the insulin/MAPK/ERK, *Wnt* and Notch pathways in a double transgenic mouse model of HBV/HBx protein to result in hepatocarcinogenesis.

### Receptor tyrosine kinase pathways

**Pathways involved with growth**

The tyrosine kinases are key regulators of cellular proliferation, differentiation, survival, metabolism, migration and cell cycle control.[70,71] Binding of insulin-like growth factors (IGF), epidermal growth factor (EGF), hepatocyte growth factor (HGF/c-MET), transforming growth factor (TGF), basic fibroblast growth factor (bFGF), platelet-derived growth factor (PDGF), and vascular endothelium growth factor (VEGF) to their corresponding receptors initiates and activates signalling cascades that promote growth and differentiation. In the context of liver regeneration following an insult, these pathways are upregulated, resulting in aberrant signaling affecting multiple pathways,[72] promoting cancer initiation and progression. Downstream, the intracellular mediators of these pathways are the Ras-mitogen-activated protein kinase (MAPK) or extracellular signalling regulated kinase (ERK), phosphatidylinositol 3-kinase (PI3K)/Akt kinase signalling pathways and JAK/STAT pathways that induce transcription of cell-proliferating genes *via* proto-oncogene *cFos* and transcription factor activator protein (AP-1).[73] Both IGF-I and IGF-II (increased expression in 12 - 44% of HCC) acting through the IGF-1 receptor (IGF-1R) are involved in the development and progression of HCC.[74] Similarly, EGFR, HGF and c-Met (a transmembrane tyrosine kinase) are implicated in aggressive HCC, associated with a poor prognosis.[75,76]

**Pathways involved with angiogenesis**

HCC is a highly vascular tumour with high metastatic potential. This is partly due to activation of VEGF (through VEGFR2), PDGF (through FGFR-1) and bFGF signalling pathways involved in neovascularisation, invasion and metastases.[77-79] High levels of VEGF are associated with postoperative recurrence and, therefore, poor prognosis in HCC.[80,81] Notably, bFGF intersects with VEGF to synergistically activate angiogenic pathways,[82] suggesting that it may indeed be a target for drug resistance against VEGF-targeted therapies. Furthermore, high preoperative serum bFGF levels are predictive of invasive tumour and early postoperative recurrence in patients undergoing resection, making this a potentially useful clinical biomarker.[79] These pathways can be inhibited by sorafenib; it is the only multi-tyrosine kinase inhibitor that targets VEGFR 1-3, PDGFR-β, c-kit, Flt3 and p38, and remains the only one approved for use in clinical practice for unresectable HCC. Sorafenib, however, confers only a 2 - 3-month survival benefit, highlighting the critical need for new therapies in this group of patients.[79,83] Newer trials have been designed that target either multiple tyrosine kinase inhibitors (TKIs) simultaneously or specific TKIs such as c-MET inhibitors or TGFβR in HCC sub-populations, with promising early results.[84]

**Pathways involved with cell differentiation**

***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. Notwithstanding, 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.[85,86] Mechanisms of *Wnt* activation include somatic mutations of CTBBB1, 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.[87] 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.[88] 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 withnormal liver tissues.[89]

**Hedgehog**

Activation of Hedgehog signalling was shown to be oncogenic 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.[90] 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).[91,92] 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,[91] particularly as an inducer of apoptosis.[93] Other mechanisms by which Hedgehog is oncogenic include the activation of MMP-9 through ERK.[94] A key role of Hedgehog activation is that it is an inducer of radiation-induced liver fibrosis, which can be targeted with inhibitors to radiosensitise tumours prior to radiotherapy.[95]

## 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 (PDL-1) and low expression of co-stimulatory CD80 and CD86,[96,97] and downregulate MHC molecules and dendritic cell activation,[98,99] thus curtailing their cytotoxic ability. In HCC specifically, there is immune exhaustion, typified by enhanced expression of co-inhibitory molecules PDL-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),[100] including decreased expression of effector cytokines, which limits cytotoxic effectiveness.[101] 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.[102] Other tumour evasion/escape strategies include the recruitment of immunosuppressive T-regulatory cells (T-regs) and monocyte-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.* [103] 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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