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► **To cite this version:**

Sandra Rebouissou, Jean-Charles Nault. Advances in molecular classification and precision oncology in hepatocellular carcinoma. *Journal of Hepatology*, 2020, 72 (2), pp.215 - 229. 10.1016/j.jhep.2019.08.017 . hal-03489688

HAL Id: hal-03489688

<https://hal.science/hal-03489688>

Submitted on 21 Jul 2022

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Advances in molecular classification and precision oncology in hepatocellular carcinoma

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Keywords: liver cancer, mutation, hepatitis B, targeted therapy, hepatocellular adenoma, malignant transformation

Financial supports: This work was supported by Institut National du Cancer (INCa) with the International Cancer Genome Consortium (ICGC LICA-FR project) and NoFLIC projects (PAIR HCC, INCa and ARC), INSERM with the « Cancer et Environnement » (plan Cancer), MUTHEC projects (INCa) and the HECAM project (BPI). The group is supported by the Ligue Nationale contre le Cancer (Equipe Labellisée), Labex OncoImmunology (investissement d'avenir), grant IREB, Coup d'Elan de la Fondation Bettencourt-Shueller, the SIRIC CARPEM and Fondation Mérieux.

Author contributions:

Drafting of the manuscript, revision of the manuscript and approval of the final version of the manuscript: JCN, SR

Conflicts of interests: No conflicts of interests

Acknowledgments: we thank Barkha Gupta for the critical reviewing of this manuscript

Abstract

Hepatocellular carcinoma (HCC) arises from hepatocytes through the sequential accumulation of multiple genomic and epigenomic alterations resulting from a Darwinian selection. Genes from various signaling pathways such as telomere maintenance, Wnt/ β -catenin, P53/cell cycle regulation, oxidative stress, epigenetic modifiers, AKT/mTOR and MAP kinase are frequently mutated in HCC. Several subclasses of HCC have been identified based on transcriptomic dysregulation and genetic alterations that are closely related to the risk factors, pathological features and prognosis. Undoubtedly, integration of data obtained from both preclinical models and human studies can be helpful in accelerating the identification of robust predictive biomarkers of response to targeted biotherapy and immunotherapy. The aim of this review was to describe the main advances in HCC in term of molecular biology and how this knowledge could be used in clinical practice in the future.

Key points:

- 1) Single nucleotide polymorphisms of *PNPLA3* (rs738409), *TM6SF2* (rs58542926) and *HSD17B13* (rs72613567) modulate the risk of chronic liver disease and HCC development, mainly in patients with alcoholic and non-alcoholic fatty liver disease
- 2) Telomerase reactivation is one of the key events in malignant transformation of cirrhotic hepatocytes. The main mechanisms of telomerase reactivation were: hot spot mutations in the promoter of *TERT*, *TERT* amplification, *TERT* translocation and, in HBV related HCC, viral insertion in the promoter of *TERT*
- 3) Each HCC is a unique combination of somatic genetic alterations. The median rate of protein altering mutations per tumor was 50 to 70 including mutations in 2 to 6 driver genes.
- 4) Several types of tumor heterogeneity have been described in HCC developed on cirrhosis: intra-tumor heterogeneity and inter-tumor heterogeneity due to multifocal independent carcinogenesis and/or intra-hepatic metastasis
- 5) Genetic features of HCC are strongly linked with risk factors, histological and clinical features. Several genomic alterations are candidate predictive biomarkers of response to targeted therapy and immunotherapy.

Introduction

Despite several advances in the management of hepatocellular carcinoma (HCC), the prognosis of patients with HCC remains poor mainly due to the high rate of diagnosis at advanced stages[1]. Several tyrosine kinase inhibitors (sorafenib, lenvatinib, cabozantinib and regorafenib) or monoclonal antibody directed against VEGFR2 (ramucirumab) have shown to improve survival when used as first line (sorafenib and lenvatinib) or second line (regorafenib, cabozantinib and ramucirumab) in patients with advanced HCC[2–6]. However, these treatments are associated with adverse events and have a limited efficacy with frequent primary or secondary resistance. More data on pathogenesis of HCC are required in order to develop new efficient therapies to increase survival of HCC patients[7].

Our knowledge of liver carcinogenesis has increased thanks to the dissection of the mechanisms of development of cirrhosis and the understanding of natural history of risk factors such as chronic viral infection[7]. Moreover, the development of mouse model of HCC has helped to recapitulate the molecular pathway of the liver carcinogenesis and the relation with microenvironment and immunity[8]. Major advancements in our knowledge of molecular profiling and genetic defects have been made possible with the advent of next generation sequencing [9–13] which has thrown insight into the frequent somatic/genetic alterations in driver genes and the main signaling pathways dysregulated in HCC [14].

In this review, we aim to describe the major recent advances in genomic studies in the field of HCC and how these studies could be utilized in clinical practice in the future..

1. Genetic predisposition and mutational processes involved in HCC development

- *Genetic predisposition*

Single nucleotide polymorphisms (SNPs) are genetic variants present in the general population that could influence the occurrence, severity and the clinical behavior of human diseases. Compared to germline mutations that are rare, constitutional genetic alterations associated with a high odds ratio of diseases development, SNPs are observed usually in more than 5% of the population and, taken alone, only modestly predispose to disease development (Odds ratio most of the time below 5)[15]. Several approaches have been developed to identify SNP associated with disease development. The first one is a candidate-based approach that test target SNP based on biological hypothesis and analysis of the literature. Several SNPs in genes belonging to inflammatory pathways (*TNF alpha*, *IL1 Beta*, *IL10*, *TGF Beta*, etc...), iron metabolism (*HFE1*), oxidative stress pathways (*GSTM1*, *SOD2*, *MPO*, etc...), DNA repair mechanisms (*MTHFR*, *TP53*, *MDM2*, etc...) were found associated with HCC occurrence in the literature[15–19]. However, most of these studies used a case/control approach and only few of SNPs have been validated externally using a prospective approach in cirrhotic patients screened by ultrasonography every 6 months in order to bypass the usual bias of a case/control study[15]. Another way to identify genetic variants is based on an agnostic approach using genome wide association study (GWAS) that tests the association between thousands of SNPs in the genome and the occurrence of a disease (Figure 1). GWAS has identified *PNPLA3* rs738409 and *TM6SF2* rs58542926 SNPs linked with the development of Alcoholic Liver Disease (ALD) and Non-Alcoholic Fatty Liver Disease (NAFLD) and their progression to cirrhosis[20,21]. *PNPLA3* rs738409 is a coding variant (I148M) involved in lipid metabolism and *TM6SF2* rs58542926 in fatty acid accumulation in hepatocytes[22–24]. Subsequent studies have showed that *PNPLA3* rs738409 and *TM6SF2* rs58542926 are associated with a higher risk of HCC development according to the etiologies of the underlying liver diseases[23,25]. If the association with HCC development on ALD or NAFLD seems consistent among studies, the association with HCC risk in hepatitis C is still debatable[26,27]. Recently, a loss of function variant (rs72613567) of *HSD17B13*, coding for a hepatic retinol deshydrogenase and protective against the development of ALD and NAFLD, was identified by GWAS [28,29]. We recently showed that this variant was also protective in the development of HCC on ALD. However, more data is needed to understand the mechanisms by which *HSD17B13* (rs72613567) protects from chronic liver diseases and carcinogenesis[30]. Currently, there is no data that suggest a potential implementation of SNPs in clinical practice. Overall, the odds ratio of the current SNPs seems to be too low to stratify patients according to the risk of HCC development for intensive screening and the incremental effect of SNPs compared to clinical predictive features is weak[15]. However, combination of several SNPs (polygenic approach) with clinical features should be adopted in the future in order to refine prediction of risk factors, screening methods and chemopreventive treatment modalities. **Currently, liver ultrasonography every 6 months is the standard method for HCC screening in the cirrhotic patients. However, usefulness of HCC screening for low-HCC risk population such as hepatitis B patients or patients with NASH without cirrhosis remains debatable. Recently, in HBV patients, clinical scoring system such as PAGE B has been proposed to identify patients at risk of developing HCC.[31]. Polygenic approach in conjunction with the clinical features could be tested to refine HCC risk score already developed in non-cirrhotic HBV patients or to identify the non-cirrhotic patients with NASH at risk of HCC development.**

- *Mutational signatures and HCC development*

Another way to understand the mechanisms that shape tumor development is to study the mechanisms of accumulation of mutations in DNA during the lifetime of malignant hepatocytes[32]. Each tumor genome bears the molecular fingerprints of the exogenous (mainly exposition to carcinogens such as tobacco, ultraviolet etc...) and endogenous processes (such as defect in DNA repair mechanism, age etc...) in DNA[33]. It helps to understand the combination of processes that induces the malignant transformation of hepatocytes (Figure 1). The type of substitution in the trinucleotide context as well as the small insertion & deletion and chromosomal rearrangement recapitulates the main mutational signatures in carcinogenesis[34,35]. Major mutational processes were observed in more than 50% of HCC and were related to aging (deamination of methylated cytosines for signature 1 and unknown mechanism for signature 5), liver specific signature (signature 16-only observed in primary liver tumors) and exposure to polycyclic aromatic hydrocarbons including but not restricted to tobacco (signature 4 observed in other solid tumors related to tobacco)[10,11,13,36]. In a small percentage of tumors, sporadic mutational signatures have been identified such as signature 24 that is pathognomonic of exposure to aflatoxin B1 a mycotoxin that contaminates culture in Africa[36]. There is also rare type of HCC that harbored mutational signature of mismatch repair deficiency (1%, signature 6)[10]. Interestingly, a subgroup of HCC, mainly from Asia, harbored A:T to T:A transversions preferentially in a CTG trinucleotide context (signature 22) that is a hallmark of exposure to aristolochic acid. Aristolochic acid is present in plants from the aristolochia species that are widely used in traditional Asian medicine[37–39]. Interestingly, analysis of mutational signature has helped to identify new risk factors of HCC development such as aristolochic acid in Asia or have provided a biological proof to the epidemiological evidences that suggested tobacco as a risk factor of HCC development. However, the mechanism underlying the development of some sporadic mutational signature (signature 17 and 23) are still unknown and need further research.

All these mutational signatures have been described initially in liver tumor. Recent studies have shown the presence of sub-clonal somatic mutations in the non-tumor liver highlighting the occurrence of mutations in known cancer driver genes in non-malignant hepatocytes due to aging, exposure to carcinogens and chronic liver damage[40,41]. However, the potential usefulness of the detection of these sub-clonal mutations in the cirrhotic liver to predict HCC occurrence is currently unknown.

Apart from being informative on etiology, mutational signatures can be used for precision oncology as biomarkers to guide treatment. This was particularly well documented for cancers with DNA repair deficiency that harbored particular mutational signatures depending on the affected DNA repair pathway. These HCC can benefit from different therapies based on their particular defects using either DNA damaging agents such as PARP inhibitors that induces synthetic lethality) or immunotherapies[42].

In HCC, repair-deficient tumors are exceptional, however, a small subgroup of HCC (7%) harboring aberrant activation of cyclin A2 or E1 was recently associated with a unique signature of structural rearrangements involving hundreds of tandem duplications and templated insertions[43]. While this subgroup of HCC does not harbor a DNA repair defect, it shares a common signature of genomic instability with *BRCA1*-altered tumors and thus may be a good candidate for PARP inhibitor based therapies that have demonstrated clinical benefit in *BRCA1*-deficient tumors[44].

2. Mechanisms of malignant transformation

- *On cirrhosis*

In the setting of chronic liver disease, persistent liver injury due to chronic inflammation and exposure to carcinogens induces repeated cycles of cell necrosis followed by proliferation. This results in telomere shortening due to absence of telomerase activity in the adult liver[45]. Thus, cirrhosis is composed of nodules of senescent hepatocytes with short telomeres. Therefore, mice models with short telomeres are prone to develop cirrhosis when exposed to chemical or genetic liver injury[46]. However, telomerase reactivation in these mice, promoted malignant transformation of hepatocytes by evading telomere shortening and replicative senescence [47]. Similar results were observed in human HCC where 95% of the tumors harbored a re-expression of the telomerase reverse transcriptase (TERT), the limiting factor of the telomerase complex[48,49]. The different mechanisms of telomerase reactivation were: hot spot mutations in the promoter of *TERT* (40 to 60% of HCC), *TERT* amplification (5%), *TERT* translocation (2 to 3%) and, in HBV related HCC, viral insertion in the promoter of *TERT* (10%)[11,43,50,51]. The multistep process of cirrhosis could be described as follows: development of premalignant lesions such as low-grade dysplastic nodules and high-grade dysplastic nodules followed by malignant transformation into early HCC. Therefore, telomerase reactivation is required in the early step of carcinogenesis in cirrhosis and *TERT* promoter mutations are the only recurrent mutations in the driver genes observed in both low grade and high-grade dysplastic nodules (between 5 to 20% of mutations) and up to 60% of early HCC's [52,53]. These results indicate that *TERT* is a gatekeeper gene which is mutated very early in liver carcinogenesis to allow reactivation of telomerase in order to bypass senescence of hepatocytes in cirrhosis. Moreover, transcriptomic analysis of premalignant nodules in cirrhosis and early HCC's have highlighted that activation of MYC, early in the course, could be involved in the process of malignant transformation[54] whereas activation of other signaling pathways such as TGF β , WNT or NOTCH occurred later during hepatocarcinogenesis[55].

Even an expert pathologist could face difficulties in differentiating premalignant lesions from early HCC on cirrhosis[56]. In this setting, further research is needed to study the potential role of *TERT* promoter mutations as predictive biomarkers of malignant transformation of premalignant nodules and to better dissect the molecular profile of premalignant lesions and early HCC in order to propose a new nosology of early lesions based on genotype/phenotype correlation.

- *On non-cirrhotic liver*

HCC development in normal liver is a rare event (less than 10% of the cases) but is a pure model that allows the identification of direct mechanisms of liver carcinogenesis outside the background noise of cirrhosis. The first road of carcinogenesis in normal liver is related to viral infections. Hepatitis B virus (HBV) is a DNA virus that could stay into the nucleus of hepatocytes in a circular covalently closed form but could also integrate into human DNA[57]. The occurrence of HBV related HCC in normal liver (without fibrosis) is due to the action of viral oncoprotein such as HbX and to a mechanism called insertional mutagenesis[57]. Viral insertion near a cancer gene could modify the expression or function of the gene and promote malignant transformation in the absence of cirrhosis. The main genes recurrently targeted by HBV insertions are *TERT*, *MLL4*, *CCNA2* and *CCNE1*[51,58,59]. However, it should be noted that viral insertional mutagenesis is not restricted to HBV related HCC developed in normal liver but it could also be seen in HBV related HCC developed on cirrhotic liver.

We recently described HCC developing in normal liver due to recurrent viral insertions of adeno-associated virus type 2 (AAV2)[60]. AAV2 is a monostrand defective DNA virus which gets inserted in the human DNA. It was previously considered as a non-pathogenic infection observed in more than the half of the population. Recurrent clonal viral insertions of AAV2 in

driver genes such as *TERT*, *MLL4*, *CCNE1*, *CCNA2* and *TNFSF10* were observed in a subset of HCC developed on normal liver without other etiologies[60]. These results suggest that AAV2 could lead to HCC development in normal liver in rare cases.

Interestingly, a subset of HCC developed on normal liver was characterized by activation of cyclin gene (*CCNE1*, *CCNA2*) either due to AAV2 or HBV insertion or due to chromosomal rearrangements that induced accumulation of duplications and templated insertions in the genome responsible for the overexpression of *TERT*[43].

Finally, the last road known to lead to HCC development in normal liver was the malignant transformation of hepatocellular adenomas (HCA)[61]. HCA developed mostly in normal liver in young women taking oral contraception and could rarely be complicated by transformation into HCC (5% of surgical series). Several genetic subgroups of HCA have been described and were linked with patho-radiological features and the risk of complications: HCA with inactivating mutations of *HNF1A*, HCA with mutations of *CTNNB1* in exon 3 or in exon 7 or 8, inflammatory HCA and sonic hedgehog HCA[62]. 10 to 15% of HCA harbored an activating mutation of *CTNNB1* (coding for B-catenin) in exon 3 and has a high risk of malignant transformation[63]. Interestingly, reactivation of telomerase due to mutations of the *TERT* promoter is required in a second step to foster a full malignant transformation of HCA[64].

3. Molecular classification of Hepatocellular carcinoma

- *Mutational, transcriptomic and epigenetic landscape*

HCC is a complex disease arising from hepatocytes through the sequential accumulation of multiple genomic and epigenomic alterations that have undergone Darwinian selection. Next generation sequencing has allowed to draw the precise genetic landscape of HCC and identified a median rate of approximately 50 to 70 protein altering mutations per tumor[10,13,65], with similar mutational burden across the different stages from BCLC 0/A to advanced HCCs (BCLC B/C)[30]. The vast majority of these mutations are passengers, they accumulate randomly and are not involved in carcinogenesis while only a few of them, about 2 to 6, are considered as functional "driver" mutations and alter key signaling pathways leading to selective advantage. Advanced computational methods for distinguishing between driver and passenger mutations[66] have allowed to identify the main mutated genes causally implicated in HCCs that can be classified in six major biological pathways including telomere maintenance, Wnt/ β -catenin, P53/cell cycle regulation, AKT/mTOR, MAP kinase, epigenetic modifiers and oxidative stress (Figure 1)[9–13,50,65,67]. The most prevalently mutated genes are *TERT* (promoter), *TP53*, *CTNNB1*, *AXIN1*, *ARID1A* and *ARID2* while the others are mutated in less than 10% of the cases (Figure 1). The interplay between mutated driver genes, also defined as epistatic interactions, is a major determinant of the carcinogenesis process. Indeed, in cancer, some mutations tend to co-occur suggesting that they cooperate together in tumor development while mutual exclusivity is usually explained either by the functional redundancy of these mutations or by their deleterious effect when arising simultaneously. In HCC, three main clusters of positive epistatic interactions have been described, the first one between *CTNNB1* mutations and *TERT* promoter, *ARID2* and *NFE2L2* mutations, the second one between *AXIN1* mutations and *RPS6KA3* and *ARID1A* mutations and the third one between *TP53* mutations and *KEAP1*, *TSC2* mutations and *CCND1/FGF19* amplification[10] (Figure 2). By contrast, a negative interaction was identified between *CTNNB1* mutations and *AXIN1* and *TP53* mutations[10] (Figure 2).

Epigenetic dysregulation also plays a crucial role in hepatocarcinogenesis by altering gene expression through various mechanisms including modifications in DNA methylation, histones, chromatin remodeling and changes in levels of small (microRNAs) and long (lncRNAs) non-coding RNAs. This is further highlighted by the frequent genetic alterations occurring in epigenetic modifiers accounting for around 20-50% of HCC cases[9–13,65]. DNA methylation and microRNA expression are so far, the most well investigated epigenetic alterations in HCC. Methylome profiling has reported aberrant promoter methylation in multiple genes some of which have been directly involved in liver tumorigenesis such as, among others, *CDKN2A* that is preferentially inactivated by promoter hypermethylation in 50% of the cases or *IGF2* that is activated through hypomethylation of its fetal promoter[13,68,69]. Some risk factors such as HBV and HCV can directly modulate DNA methylation in tumor cells and participate in carcinogenesis[7]. There are also interactions between genetic and epigenetic alterations, for example, an extensive CpG hypermethylation of multiple gene promoters was associated with *CTNNT1* mutations[13,70], likewise, *CDKN2A* promoter methylation was linked to *TERT* promoter mutations[13] and genome-wide hypomethylation was correlated with genomic instability[71]. Aberrant expression of microRNAs has been also largely implicated in the development and progression of HCC through deregulation of key cellular processes such as proliferation, apoptosis, angiogenesis, invasion, metastasis and autophagy[72]. Both genetic and epigenetic alterations participate in reprogramming gene expression in tumor cells. In HCC, multiple genome-wide gene-expression studies have allowed to identify the main regulatory networks transcriptionally altered. Pathways that are commonly aberrantly activated include the canonical Wnt/ β -catenin, TGF β , cell cycle, interferon, MYC, PI3K/AKT and MET[13,67,73–76]. Transcriptomic profiling also revealed different differentiation patterns among HCCs, some of them are well differentiated and retain a hepatocyte-like phenotype while others are less differentiated and are characterized by expression of progenitor markers such as *IGF2*, *EPCAM*, *AFP* or *KRT19*[74,75,77]. More recently, deconvolution methods applied to transcriptomic data have enabled to identify an “immune class” of HCC representing 30% of the cases and characterized by an immune gene expression signature including two clusters of HCCs with either active or exhausted immune responses[78].

Integrated transcriptional and genomic analysis has also allowed deciphering the similarities and differences between HCC, intra-hepatic cholangiocarcinoma (ICC) and the combined/mixed form of HCC-ICC, which were considered as clinically different entities. In particular, Chaisaingmongkol *et al.* have identified common molecular subtypes between HCC and ICC with similar prognosis that are specifically found in Asian patients[79]. Another recent study showed that mixed HCC-ICC are more similar to HCC while combined HCC-ICC share more common molecular features with ICC. Interestingly, nestin expression was shown as a reliable biomarker for identifying these two subtypes of tumors and may represent a potential new therapeutic target for both combined and mixed HCC-ICC[80].

- *Integrative analysis and correlation with clinical and pathological features*

Over the past decade, worldwide efforts in HCC molecular subtyping combined with analysis of the associated clinical, etiological and histopathological features have significantly refined the nosological classification of HCC leading to the definition of several subgroups. HCC are first classified in two major classes: a “proliferation class” and a “non-proliferation class”, each of which integrated several subclasses previously defined across multiple studies[13,67,73–75] (Figure 3). The “proliferation class” is associated with HBV-related etiology and includes clinically aggressive tumors, poorly differentiated with frequent vascular invasion and is characterized by an enrichment in *TP53* inactivating mutations, amplification of *FGF19* and

CCND1, as well as frequent activation of pro-survival signaling pathways including cell cycle, mTOR (hyperphosphorylation of RPS6), RAS-MAPK and MET[67,73,74,81]. This class is also characterized by a chromosomal instability and a global DNA hypomethylation profile[71,82]. The proliferation class is further subdivided in two subclasses: a “Wnt-TGFβ subclass” characterized by an activation of both TGFβ and Wnt pathways and an exhausted immune response, and a “progenitor subclass” defined by the transcriptional and protein overexpression of hepatic progenitor markers, inactivating mutations in *RPS6KA3* and *AXIN1* and hyperphosphorylation of ERK[74,75,77,78]. The main signaling pathways specifically activated in the progenitor subclass are IGF1R and AKT[67,74]. The “Wnt-TGFβ subclass” also includes a more aggressive subgroup of HCC “G3” distinguished by a specific histological phenotype designed as “macrotrabecular massive”[77,83], the occurrence of *TSC1* and *TSC2* mutations, a more pronounced enrichment in *FGF19/CCND1* amplification as well as overexpression of genes related to the cell cycle and nucleus pore[74,77].

The “non-proliferation class” of HCC is more heterogeneous and includes less aggressive tumors, chromosomally stable, related to HCV or alcohol. They are more differentiated and tend to retain hepatocyte-like features[13,73,75,81]. This class contains at least two subclasses with, as the best defined, a subset of HCC harboring *CTNNB1* mutations leading to an activation of the Wnt/β-catenin pathway that is higher in the “G6” subclass compared to “G5” [67,74]. *TERT* promoter mutations are also more frequent in this subclass as well as hypermethylation of *CDKN2A* and *CDH1* promoters[13,74]. In addition, *CTNNB1*-mutated HCC are immunologically cold and are characterized by cholestasis, nuclear accumulation of β-catenin and positive immunohistochemical staining of glutamine-synthetase (GS)[77,78]. The second subclass belonging to the non-proliferation class named “G4” is less well defined and a subgroup of HCC inside the G4 class harbored a frequent steatohepatic phenotype and an activation of the IL6/JAK-STAT pathway defined by a positive immunohistochemical expression of the C-reactive-protein (CRP)[74,77]. The G4 subclass encompasses two others subclasses named “Polysomy 7” (Poly7) and “Interferon” (IFN) that include respectively a small group of HCC with polysomy of chromosome 7 and a group of HCC overexpressing interferon-stimulated genes with an active immune response[67,78].

4. Tumor progression and tumor heterogeneity

- *Tumor aggressiveness and mechanisms of progression*

Several molecular features have been linked with tumor aggressiveness and the risk of relapse[7]. First, several transcriptomic molecular signatures have been identified as predictive of tumor recurrence: hepatoblast like signature, stem cell signature, proliferative subclass, G3 subclasses, 5 gene score, etc...[73,74,84–86] Molecular signatures derived from the non-tumor liver also have been developed to predict late relapse related to de novo carcinogenesis on cirrhosis[87]. However, these molecular signatures have not been validated in prospective studies and are currently not used in clinical practice. Other markers of poor prognosis in patients treated by liver resection have been also identified such as mutations of *TP53*, *RBI*, *CDKN2A* or amplification of *FGF19*[10,65,88].

Data available on genomic of advanced HCC is sparse mainly due to the absence of tumor biopsy performed in advanced stages in clinical practice. A recent study has showed an enrichment in *TP53*, *RBI* and *SF3B1* mutations in advanced HCC suggesting a role of these genes in tumor progression[30]. More data are needed with longitudinal samples in order to assess the mechanisms of tumor progression across the different stages of the disease.

- *Tumor heterogeneity in HCC*

Another issue in the field of molecular biology of HCC is related to tumor heterogeneity. Several studies have shown the presence of intratumor heterogeneity with trunk mutations present in all the cells of the tumor and private mutations present in only one part of the tumor (Figure 4)[89,90]. Preliminary data in HCC have suggested that the main driver genes such as *CTNNB1*, *TP53* and *TERT* are clonal trunk mutations and are consequently ideal therapeutic target[53,90–92]. However, more data are required to clarify this using ultradeep sequencing and combination with methylation and transcriptomic analysis in order to capture the complexity of intratumor heterogeneity in large cohorts of HCC. Another type of genomic heterogeneity observed in HCC is inter tumor heterogeneity that could be related to de novo independent carcinogenesis on cirrhosis and/or intrahepatic metastasis (Figure 4). According to the study and the method used to assess tumor clonality, intra hepatic metastasis are observed in 20 to 40% of the patients whereas the rest of the tumors are related to de novo carcinogenesis on cirrhosis[65,93–95]. However, we have to underline that most of these studies have been performed on surgical samples of resection or transplantation and the results may be different in more advanced stages. Obviously, a better understanding of intra tumor and inter tumor heterogeneity are mandatory to understand their role in primary and secondary resistance to systemic treatments.

5. Precision oncology in HCC

- *Biomarkers of response to tyrosine kinase inhibitors*

Targeted therapies including Tyrosine kinase inhibitors (TKIs such as sorafenib, lenvatinib, cabozantinib and regorafenib) and anti VEGFR2 antibody (ramucirumab) have substantially improved the systemic treatment of advanced HCC. Since the approval of sorafenib as the frontline standard of care[2], four additional targeted therapy have recently demonstrated clinical benefits in phase 3 trials including lenvatinib in first-line and regorafenib, cabozantinib and ramucirumab in second-line[3–6]. However, all these drugs confer only a modest extension in median survival (2-3 months) and low response rates, which underscores the need of predictive biomarkers to improve clinical management. Several potential markers have been associated with sorafenib response, such as *FGF3/FGF4* and *VEGFA* genomic amplification, overexpression of active VEGF receptors or elevated Mapk14-Atf2[96–99] and more recently, a clinical study has linked sorafenib primary resistance to the occurrence of activating mutations in the PI3K/AKT/mTOR pathway in HCC[100]. However, currently, there are still no validated predictive factors of sorafenib response that can be used in routine patient care.

Similarly, an exploratory retrospective analysis of the RESORCE trial has reported that candidate plasma biomarkers including 5 proteins and 9 miRNAs are able to predict regorafenib response, while these findings also need further confirmation[101]. Interestingly, the phase 3 REACH-2 study confirmed the predictive value of elevated serum alpha-fetoprotein (≥ 400 ng/ml) for ramucirumab sensitivity which represents so far, the first drug in HCC with a consistent predictive biomarker applicability in daily clinical practice[6]. Other biomarkers of TKI response have also been proposed in HCC such as high levels of MET detected by immunohistochemistry as a predictor of tivantinib sensitivity[102] but this finding could not be subsequently validated in phase 3 trial[103]. This failure may be explained firstly by an inappropriate selection criterion as MET overexpression may not be a surrogate marker of oncogenic addiction and secondly by an inadequate treatment since tivantinib does not act as a MET inhibitor, as reported by multiple studies[104–106]. Alternatively, true MET inhibitors may be effective in the small subset of patients with *MET* amplified tumors as we have recently reported in liver cancer cell lines and in one patient who achieved a complete tumor response after treatment by tepotinib, a specific Met inhibitor[30,106,107].

- *Biomarkers of response to immunotherapy*

In recent years, immunotherapy and especially, immune checkpoint inhibitors have revolutionized the management of broad spectrum of malignancies. HCC is a typical inflammation-associated cancer and is known to arise in a context of immunosuppression making immunotherapy a potential attractive therapeutic option. In this line, two phase 2 studies have reported objective responses to nivolumab and pembrolizumab, two immune checkpoint inhibitors targeting PD-1, in 20% and 17% of advanced HCCs, respectively[108,109]. These results have led to an accelerated approval of these two checkpoint inhibitors by the FDA for the treatment of advanced HCC in second-line. However, recent press release reported that the phase 3 randomized controlled trial comparing nivolumab versus sorafenib in first line as well as the phase 3 randomized controlled trial comparing pembrolizumab versus placebo in first line did not reach their prespecified endpoints. It suggests that additional data are required to identify patients who will benefit from immunotherapy. In both the phase 2 studies, PD-L1 expression status on tumor cells was not predictive of the response rates, thus, alternative predictive biomarkers remain to be identified. Apart from PD-L1 expression, in other cancer types, several biomarkers have been proposed such as high levels of tumor-infiltrating lymphocytes, especially effector cells, an increased secretion of IFN- γ and other cytokines, as well as, a high somatic mutational burden and neoantigen load[110]. Interestingly, the newly identified “immune class” of HCC harbors features that closely resemble those of cancers vulnerable to checkpoint inhibitors including high infiltration of immune cells, active interferon gamma signaling and expression of PD-1 and PD-L1, making it a promising candidate for immunotherapy [78]. However, future clinical investigations will be required to confirm this hypothesis. By contrast, *CTNNB1* mutated HCCs are characterized by an immune exclusion, accordingly, a recent clinical study has shown that this subgroup was refractory to immune checkpoint blockers[100]. A recent study confirmed in a mouse model that β -catenin activation blocked T cell activity, promoted immune escape and were responsible of resistance to immunotherapy[111]. In light of these recent findings it would be interesting to reanalyze the negative phase 3 nivolumab and pembrolizumab trials by excluding *CTNNB1* mutated HCC.

Hence, development of efficient immune checkpoint-inhibitor-based therapy in HCC is still challenged by the identification of accurate predictors of response. An additional way to increase the efficacy of anti PD1/PDL1 antibodies is their combinations with either tyrosine kinase inhibitor such as lenvatinib, an anti VEGF antibody such as bevacizumab or an anti CTLA4 antibody such as tremelimumab. The first results of phase 2 released at the ASCO and ESMO meetings seem encouraging in term of tumor response and phase 3 clinical trials are currently on-going.

- How to modelize response to target therapy?

Despite the significant success of targeted therapies in the management of many cancers, HCC is one of the malignancies that have so far benefited the least from these advances. This lack of progress relies in part on the scarcity of HCC tissues, especially for advanced stages, which represents a major barrier to understand the relationship between tumor biology and the therapeutic response. In this context, the development of relevant preclinical models is particularly needed. In cancer, the most commonly used models include tumor-derived cell lines and patient-derived tumor xenografts (PDXs), as they have been shown to retain most of the common molecular features found in primary tumors[112–117]. Over the past decade, several pan-cancer large-scale pharmacogenomics screenings have been conducted on monolayer-cultured cancer cells[112,113,116]. These studies on large set of cell lines are

representative of tumor diversity and combined with deep molecular characterizations have proven to be powerful tools for molecular prediction of drug response and biomarker discovery[112,113,116]. In HCC, until now the vast majority of the *in vitro* pharmacological studies have been scattered in the literature and restricted to an insufficient number of cell lines to capture HCC diversity. To fill this gap, we recently performed an extensive multi-omic characterization of the most widely used liver cancer cell lines (LCCL) including 34 models, combined with a pharmacological screen of 31 anti-cancer agents[107]. This study showed that the current available LCCL faithfully recapitulate protein, mutational and transcriptional signatures of the proliferation class of HCC and may serve as platform to identify molecular contexts associated with drug vulnerabilities that may help to guide clinical trials[107]. This dataset is publicly available through an online portal with data visualization (<http://lccl.zucmanlab.com>) and provides a rich resource that may help to accelerate research in liver cancer (Figure 5). However, cell line models for the non-proliferation class are still lacking, probably due to the poor success rate of establishment[118,119]. Large-scale functional screenings using shRNA or CRISPR/CAS9 have also emerged as promising methods to draw a comprehensive mapping of cancer genes and identify potential new therapeutic targets, but for now, very few or no HCC cell lines were included in these studies[120,121]. Although tumor-derived cell lines have the advantage of being easy to grow, inexpensive and suitable for high throughput studies, two-dimensional culture condition does not fully recapitulate the native 3D environment of tumor cells and may influence drug response. PDTXs mimic primary tumors more closely than 2D culture, to date, a total of 116 PDTX generated from HCC have been reported in the literature and all the available clinical annotations, molecular and drug response data have been regrouped in the PDXliver database (<http://www.picb.ac.cn/PDXliver/>)[122]. However, this resource is limited by the small number of models with drug response data (only 26) and by an incomplete molecular characterization (Figure 5)[122]. Moreover, the relatively low engraftment efficiency and long engraftment periods (several months) of HCC is still limiting for large-scale drug screenings[123–125]. Alternatively, liver cancer organoid cultures generated from fresh tumor tissues also emerge as valuable models that mimic more closely parental tumors than 2D culture and can be used in large drug screenings (Figure 5)[126,127]. The major drawback of both organoids and 2D culture cell lines is that they lack immune system and stromal components, consequently, they are not suitable for evaluating therapies targeted against tumor microenvironment such as anti-angiogenic, the most effective agents to date in the systemic treatment of HCC, or immunotherapies which cannot be explored either in PDTX models (Figure 5B). Dozens of HCC models in immunocompetent mice have also been developed including spontaneous, chemically-induced and genetically engineered models (Mouse Tumor Biology database <http://tumor.informatics.jax.org>) (Figure 5)[128–130]. While they are useful for studying basic tumor biology, their use as relevant preclinical platform to evaluate the therapeutic response in HCC is still limited by the lack of systematic cross-species comparative molecular analyses[131–133].

- *New biomarkers*

While, tumor tissue is still the gold standard source for identifying cancer specific biomarkers, liquid biopsy has become a promising alternative non-invasive procedure allowing the isolation and detection of cancer-derived subcellular components that are released in biological fluids such as blood[134]. Thus, it enables to overcome the difficulty in obtaining tissue biopsies, as in HCC. Several circulating biomarkers can be detected in liquid biopsies such as DNA, tumor cells, microRNAs and exosomes that are secretory vesicles containing nucleic acids and proteins[134]. Hence, liquid biopsy has become an appealing source of biomarkers for several

applications in cancer such as diagnosis, prognosis and for prediction of response to treatment[134]. Liquid biopsy also has the advantage of allowing longitudinal monitoring of tumors and may capture the spatial and temporal intra-tumor heterogeneity that is not feasible with traditional tissue biopsy. However, although liquid biopsy is likely to revolutionize the clinical management of cancers, this technology is still a research field, which is currently facing multiple technical issues that need to be solved, in particular to improve its sensitivity and specificity before future clinical applications.

Conclusion

Although substantial progress has been made in the treatment of HCC, the implementation of an effective precision medicine is still hampered by several obstacles. Firstly, there is still a lack of reliable biomarkers to predict response to current therapies and newer more effective treatments are needed. Since no clear oncogenic addiction loops have been reported in HCC, a better understanding of the epistatic interactions between mutated driver genes may help to develop more potent combination-based therapies. Also, a more detailed characterization of intra and inter-tumoral heterogeneity using longitudinal samples and single cell approaches may provide new insights into the resistance to treatment. Moreover, much more efforts have to be undertaken in developing newer relevant preclinical models, in particular, taking into account the tumor microenvironment, especially the immune contexture that may be feasible by establishing organoid co-culture systems or PDX in mice with humanized immune system. Finally, systematic tumor biopsy and blood sampling should be done for patients with HCC and more biomarkers based clinical trials should be implemented in the future. Integration of the data collected in both preclinical models and in humans should enable to accelerate the identification of robust predictive biomarkers and ultimately improve the clinical management of HCC (Figure 5A).

Figure 1: Genetic predisposition and mutational process in HCC

We figured the multistep process of carcinogenesis on cirrhosis as well as the risk factors of HCC development on normal liver. We also underlined the main SNP in genes promoting cirrhosis and HCC development (in red, *PNPLA3* and *TM6SF2*) and protecting from cirrhosis and HCC development (in blue, *HSD17B13*) as well as genes with SNP potentially involved in HCC development but that deserve further validation (in grey). We also represented the mutational process operative in malignant hepatocytes as well as the main somatic genetic alterations in signaling pathways involved in liver carcinogenesis.

Figure 2: Major epistatic interactions between mutated driver genes identified in HCC

Positive interaction indicates a co-occurrence between the mutated genes, while negative interaction indicates mutual exclusivity between the mutated genes.

Figure 3: Integrative molecular and pathological classification of HCC

The main molecular subclasses of HCC shown are derived from transcriptomic based classifications previously reported in five studies (Lee, Boyault, Chiang, Hoshida and TCGA) “Unannot”: unannotated group of HCC from Chiang’s classification without specific features. For each subclass, major associations with pathological, immunohistochemical (IHC), genetic, epigenetic, immunological and clinical features are shown.

Figure 4: Tumor heterogeneity in HCC

We represented intra-tumor heterogeneity in the upper panel. As an example of spatial and temporal intratumor heterogeneity, we described a transformation of HCA in HCC with the HCC part who relapse 2 years after. In the lower panel, we figured description of inter tumor heterogeneity on cirrhosis including intrahepatic metastasis and multifocal independent de novo carcinogenesis. Intrahepatic metastasis and multifocal independent carcinogenesis could coexist in the same liver.

Figure 5: Modeling of the therapeutic response in HCC

A) Strategy to improve precision medicine in HCC based on integration of preclinical models and analyses from HCC patients. **B)** Main features of HCC preclinical models.

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Figure 1

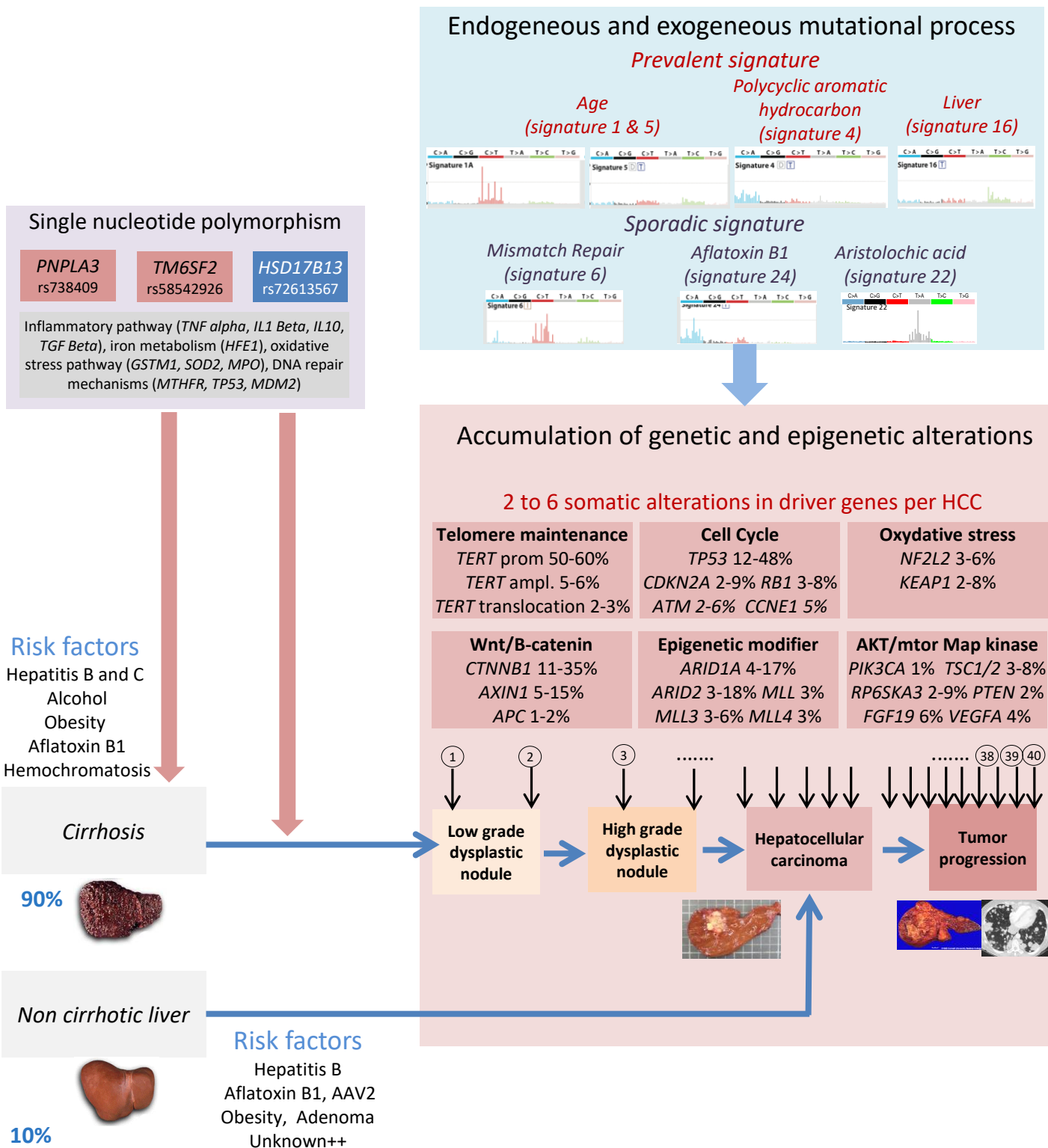


Figure 2

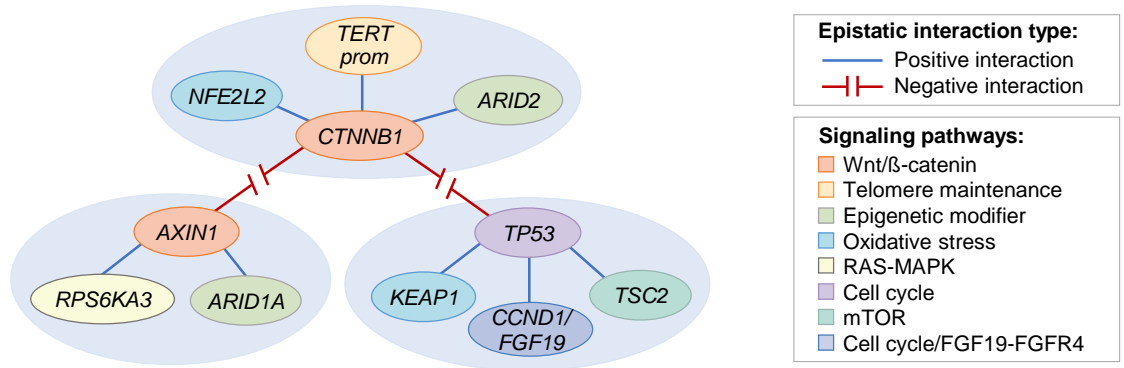


Figure 3

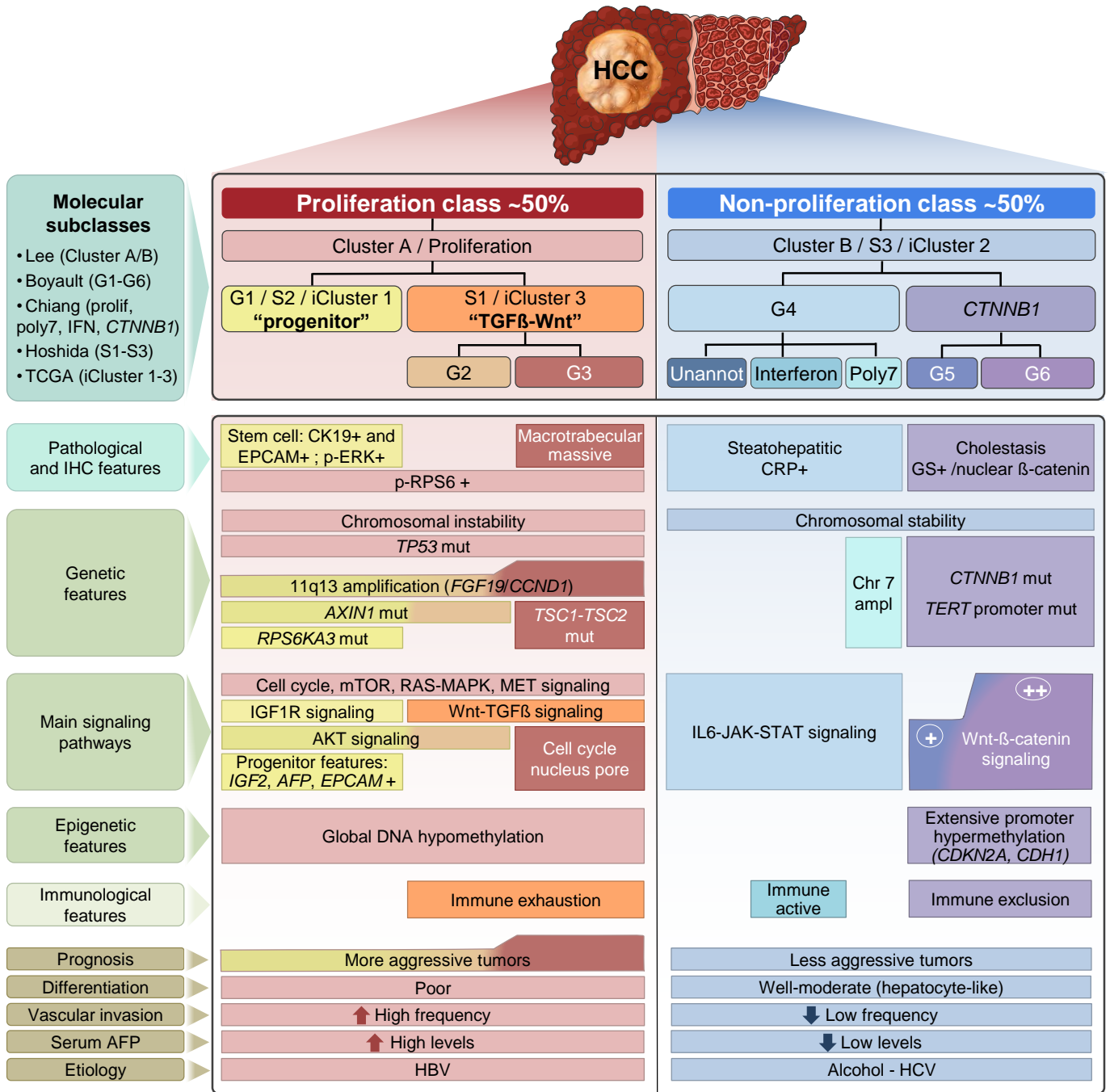
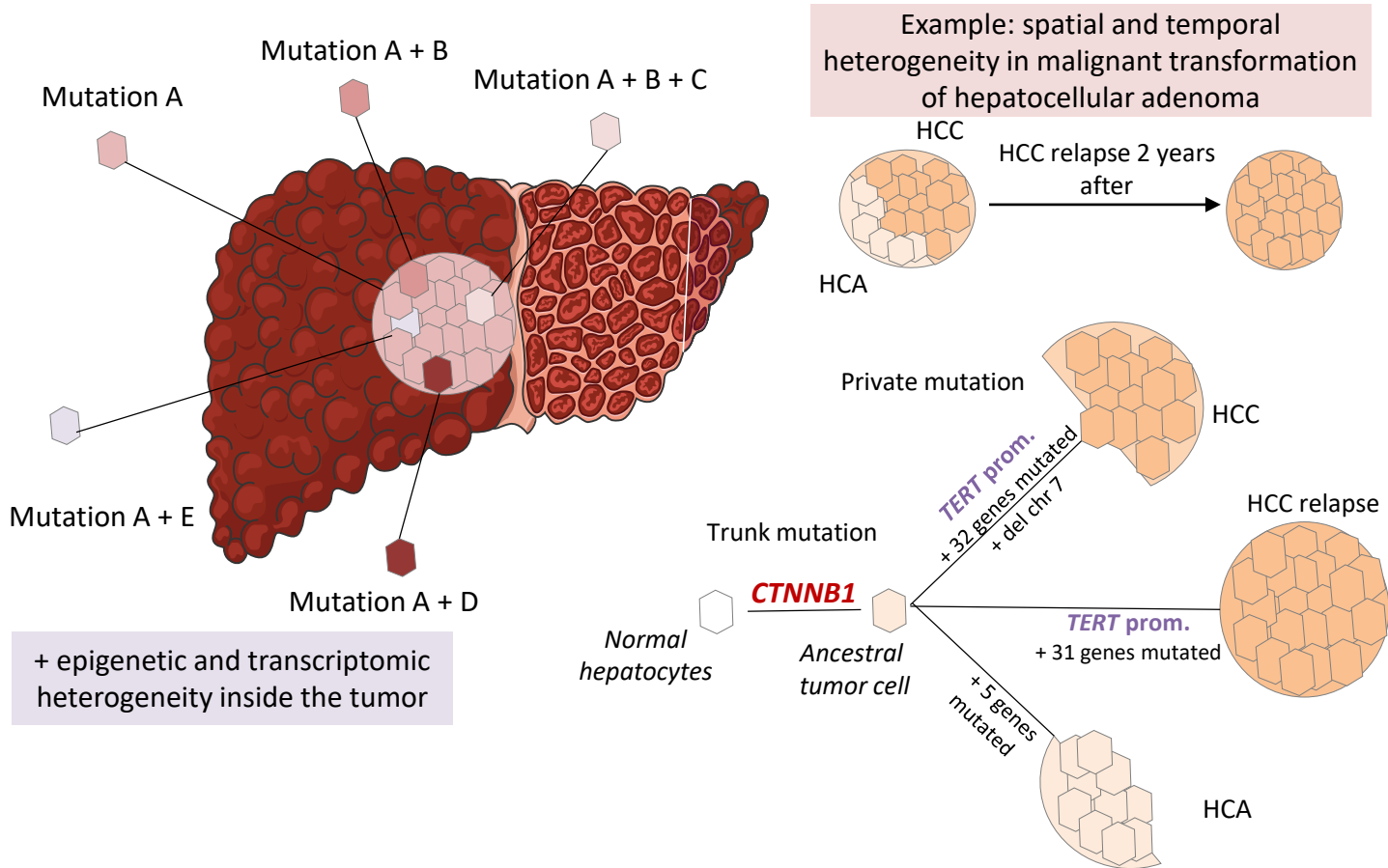


Figure 4

Intra-tumor heterogeneity



Inter-tumor heterogeneity

Intra-hepatic metastasis
Clonal metastasis with acquisition of private mutations during evolution

De novo carcinogenesis
Multifocal independent tumors of different clonal origin
Cirrhotic background

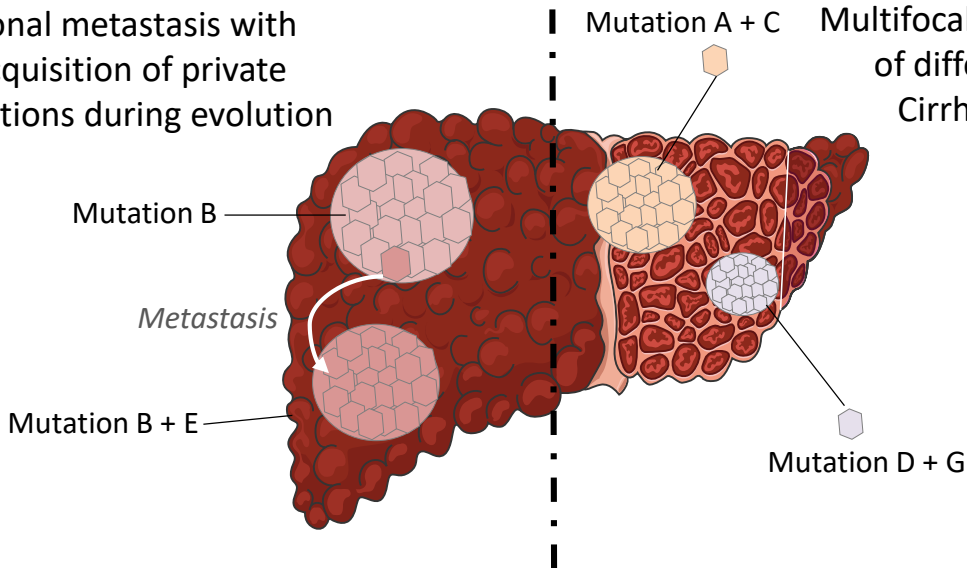
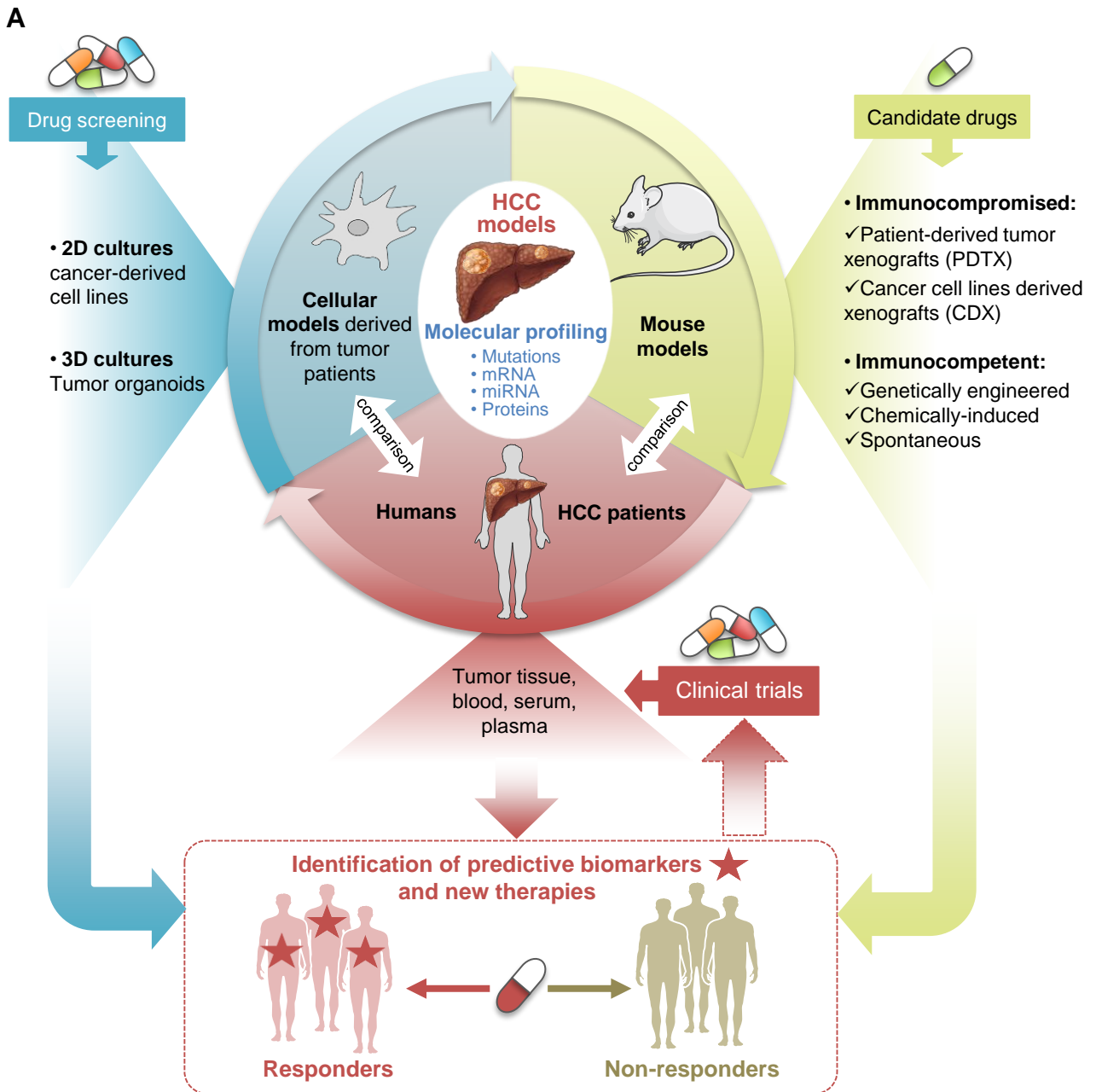


Figure 5



B

	Cellular models derived from tumor patients		Immunocompromised mice		Immunocompetent mice ^[129]		
	2D cancer-derived cell lines ^[107]	3D tumor organoids ^[126,127]	Patient-derived xenografts (PDX) ^[122]	Cancer cell line-derived xenografts (CDX)	Genetically-engineered	Chemically-induced	Spontaneous
Cost	Low	Moderate	High	Moderate	High	Moderate	Low
Time required	Low	Moderate	High	Moderate to high	High	High	High
Establishment success rate	14-29% ^[118,119]	18-26% ^[126,127]	26-35% ^[124,125]	NA	/	/	/
Ease of propagation	High	High	Moderate to difficult	Moderate to difficult	/	/	/
Suitable for high-throughput screening	Yes	Yes	No	No	No	No	No
Functional immune system	No	No	No	No	Yes	Yes	Yes
Cancer-stromal interactions	No	No	Yes	Yes	Yes	Yes	Yes
Multi-omic characterization	Yes	Yes	Incomplete ^[122]	NA	Few models ^[131,132]	Incomplete ^[131,133]	Incomplete ^[133]
Human relevance	++	+++	+++	++	Variable, model dependant ^[131]	Poor (DEN model) ^[131,133]	Not well characterized ^[133]