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Hepatocellular carcinoma: Therapeutic advances in signaling, epigenetic and immune targets

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Abstract

Hepatocellular carcinoma (HCC) remains a global medical burden with rising incidence due to chronic viral hepatitis and non-alcoholic fatty liver diseases. Treatment of advanced disease stages is still unsatisfying. Besides first and second generation tyrosine kinase inhibitors, immune checkpoint inhibitors have become central for the treatment of HCC. New modalities like epigenetic therapy using histone deacetylase inhibitors (HDACi) and cell therapy approaches with chimeric antigen receptor T cells (CAR-T cells) are currently under investigation in clinical trials. Development of such novel drugs is closely linked to the availability and improvement of novel preclinical and animal models and the identification of predictive biomarkers. The current status of treatment options for advanced HCC, emerging novel therapeutic approaches and different preclinical models for HCC drug discovery and development are reviewed here.

Key words: Liver cancer; Immunotherapy; Checkpoint inhibitors; Targeted therapy; Mouse model; Biomarker; Next-generation sequencing; Non-alcoholic steatohepatitis; Fibrosis; Clinical trial

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Core tip: Treatment of advanced hepatocellular carcinoma still represents an unmet

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medical need. Novel therapeutic options comprise new tyrosine kinase inhibitors, epigenetic modifiers and increasingly also cell therapy and immune checkpoint inhibitors and combinations of those modalities. Development of better drugs is closely linked to improved preclinical and animal models and has to be accompanied by the implementation of predictive biomarkers, which is still lacking for hepatocellular carcinoma. The current status of these aspects is reviewed in this manuscript.

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the most common primary malignant tumor of the liver, accounting for approximately 85% of all cases. It is considered to be the 6th most commonly diagnosed cancer and the 4th most common cause of cancer related death worldwide, with 2 to 3 times higher rates for men^[1]. Major risk factors are chronic viral hepatitis [hepatitis B virus (HBV) and hepatitis C virus (HCV)], aflatoxin exposure, alcohol intake, and the globally increasing high rates of obesity and type 2 diabetes^[2]. The 5-year survival rate of advanced HCC remains devastating at 1% and is the poorest of all solid cancers^[3]. Treatment of advanced stages of HCC is often limited by the underlying liver disease which is commonly accompanied by cirrhosis and end-stage liver disease with significantly impaired liver function. Due to those different etiologies and pathogenetic mechanisms, the identification of common oncogenic drivers is challenging although heterogeneous sets of mutations could be detected in HCC (Table 1), of which especially telomerase, p53, β -catenin and others were linked to distinct and prognostic HCC subtypes (Table 2)^[4,5].

Curative therapy is currently only possible in early stages by complete surgical resection or orthotopic liver transplantation, the latter being limited by availability of donor organs. Locoregional therapies [*e.g.*, transarterial chemoembolization (TACE), different ablation strategies, selective internal radiotherapy (SIRT)] are available for intermediate stages or HCCs not amenable to surgical therapy and can be applied repeatedly also for downstaging in preparation of transplantation or in an adjuvant setting prior to surgery^[6,7]. In addition, external beam radiation (EBRT) is a valuable adjuvant therapy option for small HCCs, in combination with surgery or other locoregional therapies or as a bridging option to orthotopic liver transplantation. It can also help to reduce pain in extrahepatic metastases and prolong survival after surgical resection^[8]. For further details on locoregional and non-systemic treatment options, we refer the reader to a recent meta-analysis on the management of HCC^[9].

Since the introduction of the multi-kinase inhibitor sorafenib about a decade ago, only little progress has been made in treatment of advanced HCC. In this article, we will review the current status of novel drugs for the treatment of advanced HCC including the emerging immune checkpoint inhibitor therapies. We will also highlight recent trends in identifying predictive biomarkers and establishing animal models that closely resemble the complex and diverse human pathophysiology of HCC.

CONVENTIONAL CHEMOTHERAPY AND MOLECULAR TARGETED THERAPIES

Prior to the approval of sorafenib, no standard chemotherapy regimen had been established for treatment of advanced HCC. Randomized trials and meta-analyses showed a poor response rate of different agents like 5-fluorouracil (5-FU), cisplatin, doxorubicin or hormonal therapy (*e.g.*, tamoxifen or somatostatin-analogues). The high intrinsic resistance of HCC is considered due to the high expression of efflux pumps (linked to the physiologic metabolic capacity of the liver parenchyma), altered blood flow and fibrosis as well as high expression and mutations in drug resistance genes like p53. Most tested drugs showed only modest activity with minimal improvement in overall survival but with significant toxicities in combination^[6,10].

Table 1 Known dysregulated pathways and genes in hepatocellular carcinoma with mode of action and frequency (modified from^[4,5,92])

Pathways / genes	Alteration	Frequency in HCC
AKT-mTOR-MAPK signaling		
RPS6KA3	Mutation	2%-9%
TSC1 and TSC2	Mutation or deletion	3%-8%
PTEN	Mutation or deletion	1%-3%
FGF3, FGF4 and FGF19	Amplification	4%-6%
PI3KCA	Mutation	0%-2%
Angiogenesis		
VEGFA	Amplification	3%-7%
Antioxidation		
NFE2L2 KEAP1	Mutation Mutation	3%-6% 2%-8%
Cell cycle control/tumor suppressors		
TP53*	Mutation or deletion	12%-45%
RB1	Mutation or deletion	3%-8%
CCND1*	Amplification	5%-14%
Epigenetic and chromatin remodeling		
ARID1A*	Mutation or deletion	4%-17%
ARID2*	Mutation	3%-18%
BAP1	Mutation	5% ^[117]
Immortalization/telomere maintenance		
ERT*	Promotor mutation amplification	54%-60% 5%-6%
JAK/STAT		
JAK1	Mutation	5%
Metabolic pathways		
Afamin apoptogenic protein 1, mitochondrial	Mutation	Up to 10% ^[117]
Oncogenes		
MET*	Amplification	30%-50%
MYC	Amplification	4%
TGFβ pathway		
Osteopontin	Mutation	Up to 40% ^[118]
G2/mitotic-specific cyclin-B2 Cyclin-dependent kinase 1 lymphoid enhancer-binding factor 1		
Integrin α2		
Wnt pathway		
Catenin β1*	Mutation	11%-37%
AXIN1*	Mutation or deletion	5%-15%

HCC: Hepatocellular carcinoma.

The small-molecule multi-kinase inhibitor sorafenib was the first drug to show an overall survival benefit in first-line therapy of advanced HCC (10.7 mo *vs* 7.9 mo; 6.5 mo *vs* 4.2 mo in Asian patients) in randomized controlled trials^[11,12]. Since then, only lenvatinib was able to achieve increased overall survival (in a phase 2 study) and was proven to be non-inferior to sorafenib in a recent phase 3 study, reaching a median survival time of 13.6 mo compared to 12.3 mo for sorafenib^[13,14].

Similarly, results for new drugs in a second-line setting after failure of sorafenib were mostly disappointing. Regorafenib, a derivative of sorafenib, and the novel multi-kinase inhibitor cabozantinib achieved a significant increase in overall survival in placebo-controlled trials. Regorafenib increased overall survival to 10.6 mo *vs* 7.8 mo^[15], while cabozantinib achieved overall survival of 10.2 mo *vs* 8.0 mo with toxicity similar to regorafenib^[16]. Lately the vascular endothelial growth factor receptor 2 (VEGFR-2) antibody ramucirumab proved efficacy in sorafenib pretreated patients with an alpha-fetoprotein level of ≥ 400 mg/mL^[17]. The randomized phase-III placebo controlled trial REACH-2 showed median overall survival times of 8.5 mo for ramucirumab treated patients *vs* 7.3 mo for the placebo arm [hazard ratio (HR) = 0.7; $P < 0.0001$]. This is the first study to show a significant survival benefit for a bio-marker (alpha-fetoprotein) selected subgroup of HCC.

Table 2 Summary of classification schemes of hepatocellular carcinoma (modified from^[119])

First author	Lee <i>et al.</i> ^[120]	Boyault <i>et al.</i> ^[121]	Chiang <i>et al.</i> ^[122]	Hoshida <i>et al.</i> ^[123]	Désert <i>et al.</i> ^[124]	TCGA network ^[117]
Year	2004	2006	2008	2009	2017	2017
HCC cases	91	56	91	232	1133	559
Number of subgroups	2	6	5	3	4	3
Names of classes	Cluster A/B	G1-G6	CTNNB1-proliferation	S1-S3	PP, PV, ECM, STEM	iCluster1-iCluster3
Major applied technology for molecular profiling						
Transcriptomics	X	X	X	X	X	X
Genetic Mutations		X				X
Copy number alterations			X			X
Metabolomics					X	
Epigenomics		X (CDH1 and CDKN2A)				X
Proteomics						X
Major HCC Classes with clinic-pathological features and high mutation rates						
Proliferative phenotype						
Poor outcome	A	G1, G2, G3	Proliferation	S1 + S2	ECM + STEM	iCluster 1 + 3
High AFP						
Moderate to poor differentiation						
P53						
Non-proliferative phenotype						
Good to moderate outcome	B	G5, G6	CTNNB1	S3	PP + PV	iCluster 2
Low AFP						
CTNNB1						

ECM: Extracellular matrix; PP: Periportal; PV: Perivenous; STEM: Stem/progenitor cells; HCC: Hepatocellular carcinoma.

Several other targets for inhibition of receptor tyrosine kinase function in HCC were investigated. Hepatocyte growth factor (HGF) and its receptor c-Met are commonly overexpressed in HCC and have been linked to poor prognosis and resistance to *e.g.*, sorafenib treatment^[18-20]. c-Met is targeted by several multi-kinase inhibitors like gefitinib or cobazitnib and more recently also selective inhibitors like capmatinib or tepotinib entered clinical trials but results for studies in HCC are still pending^[21-23]. Other less selective compounds with c-Met inhibition properties like crizotinib, brivanib or foretinib did not lead to significant prolongation of overall survival (OS) in phase III studies or were not investigated in HCC patients yet^[21,22,24,25].

All of these compounds are recommended for patients with preserved liver function, *i.e.*, Child-Pugh score 5, 6 and 7^[26]. Treatment options for patients with more advanced liver impairment or cirrhosis are still lacking and represent an urgent medical need. Esp. the use of sorafenib in patients with portal vein tumor thrombosis (PVTT) remains controversial^[27]. In a study with 30 patients with advanced HCC and PVTT treated with sorafenib monotherapy, a disease control rate of 33.3% was achieved, including thrombus revascularization in a small number of patients. Yet, OS and progression-free survival (PFS) still remained disappointing with only 3.1 and 2.0 mo, respectively^[28]. In combination with TACE, sorafenib was able to induce a significant survival benefit compared to TACE only in patients with type B (13 mo *vs* 6 mo) or type C (15 mo *vs* 10 mo) in a study enrolling 99 patients^[29]. Similar results were obtained in combination with radiofrequency ablation (RFA)^[30]. Still, prospective randomized controlled trials on sorafenib or regorafenib monotherapy in this setting are missing and the effect of the combination approach is probably overruling the currently available results^[27].

Different combination studies of sorafenib and other agents have been performed. Interestingly, vitamin K was shown to enhance the antitumor effects of sorafenib via reduction of expression of des- γ -carboxy prothrombin (DCP), a proangiogenic growth factor that can also trigger signaling *via* c-Met and which is commonly upregulated after sorafenib treatment^[31,32].

Combination studies with other targeted agents showed only a modest increase in survival but had significant increase in drug related adverse events or even showed a worse outcome than single agents like the sorafenib/erlotinib combination^[33]. These agents have also been investigated in earlier disease stages but could not demonstrate a survival benefit in combination with locoregional approaches like TACE or in an adjuvant setting^[7].

In summary, sorafenib and lenvatinib are options for first-line therapy of advanced HCC, while regorafenib, cabozantinib and ramucirumab can be used as second-line options afterwards.

IMMUNE CHECKPOINT INHIBITORS AND NOVEL IMMUNOTHERAPY TARGETS IN HCC

The development of immune checkpoint inhibitors like anti-CTLA4 (ipilimumab, tremelimumab) or anti-PD-1/PD-L1 (nivolumab, pembrolizumab, tislelizumab, camrelizumab, atezolizumab, durvalumab, avelumab) antibodies has dramatically changed clinical oncology nowadays and achieved sustained treatment responses in an unprecedented manner across different cancer types. Chronic inflammation due to the various underlying etiologies is a mainstay of HCC development. Different immune cell subtypes (T cells, macrophages, Kupffer cells) are currently intensively investigated to understand their role in HCC pathogenesis and to exploit them as novel and specific therapeutic approaches for this disease. Kupffer cells and CD8+ T cells in HCC have been shown to express high levels of PD-1 and PD-L1, thus playing a key role in the immune evasive phenotype of HCC. High expression of PD-L1 on tumor cells was associated with poorer outcome in HCC patients^[34-37].

Several immune checkpoint inhibitors are currently investigated in clinical trials as single agents or in combination with neo-epitope releasing locoregional therapies, epigenetic drugs or conventional targeted agents^[38].

In a phase 1 study, tremelimumab reached a clinical disease control rate of 76.4% with 17.6% of patients achieving partial response^[39]. In combination with subtotal radiofrequency ablation or chemoablation, 26.3% of patients reached partial response and a median overall survival of 12.3 mo. Responders also showed increased infiltration of CD8+ T cells and HCV positive patients had a significant reduction in viral load^[40].

Based on positive results of the phase 1/2 CheckMate-040 study, nivolumab received FDA approval as a 2nd line therapy option in HCC^[41]. An objective response rate of 20% was reached overall, and patients expressing PD-L1 on tumor cells even reached 26%. A subgroup analysis revealed disease control rates of up to 75% and a median duration of response of 9.9 mo. Results of the phase 3 CheckMate-459 study that compares nivolumab *vs* sorafenib are still pending.

Similar results were obtained for pembrolizumab in the KEYNOTE-224 study. Here, 17% of patients had partial response, 44% had stable disease and overall 77% of responding patients had durable responses of 9 mo or more. Pembrolizumab also received FDA approval as a 2nd line therapy after sorafenib therapy.

Several further studies are currently ongoing to evaluate these checkpoint inhibitors in first or second line therapy of HCC, including the HIMALAYA study that explores durvalumab alone or in combination with tremelimumab *vs* sorafenib or the IMbrave50 study that combines atezolizumab with bevacizumab *vs* sorafenib^[42].

Due to its physiologic role in clearing portal vein blood flow from potentially harmful gut content, the liver is a key immunologic organ and contains a high proportion of macrophages (Kupffer cells) and other cells of lymphoid lineage, including B and T cells as well as natural killer (NK) and NKT cells^[43]. As outlined above, HCC commonly develops on the basis of chronic inflammatory liver injury and exploiting the immunologic repertoire of the liver. The first promising results of immune checkpoint inhibitors in HCC further support this approach. Different technologies have been developed to apply an adoptive cell transfer as a therapeutic option also in HCC, including application of tumor infiltrating lymphocytes, cytokine induced killer cells or fostering neoepitope release by locoregional ablation techniques^[44-46].

Recently, chimeric antigen receptor-engineered T (CAR-T) cells yielded outstanding responses in hematologic malignancies and received FDA approval for the treatment of acute lymphoblastic leukemia^[47,48] and diffuse large B-cell lymphoma^[49,50]. In brief, T cells of patients are harvested, genetically modified, expanded and reinfused into the patient. CARs consist of an extracellular antigen recognition domain, a hinge/spacer domain, a transmembrane domain and a T cell activation domain (CD3 ζ). CARs of the 2nd and 3rd generation have additional costimulatory molecules like CD27 or CD134 in between the transmembrane and the CD3 ζ domain to

achieve prolonged T cell expansion and antitumor effects^[51]. Success of CAR-T cells in solid tumors is limited by their broader mutational load compared to hematologic malignancies and suitable tumor antigens are thus more difficult to identify. An additional hurdle is the localization of modified T cells to the tumor, which is influenced by tumor angiogenesis, low levels of chemokines to attract T cells into the tumor and, esp. in HCC, a tumor microenvironment (stroma, fibrosis) that does not permit sufficient tissue penetration of large molecules^[52]. The latter can be overcome by selective local administration like hepatic artery infusion which makes HCC an interesting option for this therapy approach and several studies using CAR-T cells were therefore initiated in HCC^[44,53] (Table 3). Interestingly, most studies use the cell surface glycoprotein glypican-3 (GPC3) as an antigen, which is highly expressed in HCC but not in other adult tissues and has been linked to poor prognosis of HCC^[54,55]. Preclinically, CAR-T cells targeting GPC3 were able to eliminate HCC cells and prolong survival of tumor-bearing mice^[56,57]. Ongoing studies also evaluate different application routes like hepatic artery infusion, systemic infusion or combination with transarterial (chemo-)embolization and the effect of lymphodepleting conditioning. Preliminary results from study NCT02395250 indicate that GPC3-CAR-T cells are safe in relapsed or refractory Chinese HCC patients. In this study, 1 partial response and 2 stable diseases were observed as best response (from 6 evaluable patients), all durable for more than one year^[58].

EPIGENETIC THERAPY WITH HDAC INHIBITORS IN HCC

Epigenetic dysregulation of gene activity is essentially involved in HCC tumorigenesis as evidenced by dysregulation of histone deacetylases *in vitro*, *in vivo* and *in situ*^[59,60]. Treatment with HDAC inhibitors (HDACi) therefore represents an attractive therapeutic option in liver cancer that addresses different molecular mechanisms compared to chemotherapy or targeted therapies to inhibit tumor cell growth and promote cell death. HDACi inhibitors commonly inhibit cell cycle progression by re-expression of p21^{cip1/waf1} in a p53-dependent manner but can also mediate alternative cell death pathways like unfolded protein response and ER stress pathways due to their non-specific acetylation of proteins also outside the nucleus^[61-65]. Although some case reports showed a positive effect of HDACi in combination with sorafenib in HCC^[66], most studies are disappointing so far. A phase 2 study using belinostat (inhibitor of all zinc dependent HDAC isoforms) in unresectable HCC showed a PFS and OS of 2.6 and 6.6 mo, respectively^[67]. The SHELTER study investigated resminostat (oral pan-HDACi with predominant activity against HDAC1, 2 and 3) in combination with sorafenib in a 2nd line setting of advanced HCC and demonstrated an OS of 8.0 mo, while monotherapy resminostat reached only 4.1 mo^[68]. Interestingly, this combination was also used in a 1st line setting in Asian patients but did not provide evidence for an OS benefit over sorafenib^[69].

These studies, like others investigating tyrosine kinase inhibitors, usually enroll patients with progressive, unresectable, locally advanced or metastatic HCC with an overall poor prognosis that limits the chance of achieving PFS or OS advantages^[70]. Yet, epigenetic therapies may be able to overcome such hurdles and even enhance the results of immune checkpoint inhibitors in HCC. Epigenetic targeting with the enhancer of zeste homolog 2 (EZH2), a histone-lysine-N-methyltransferase, inhibitor 3-Deazaneplanocin A (DZNep) and the DNA methyltransferase 1 (DNMT1) inhibitor 5-azacytidine reactivated transcriptionally repressed chemokines genes and augmented T cell trafficking to the tumor^[71]. Consequently, epigenetic pretreatment may lead to priming of so-called immune cold tumors also in HCC^[71,72].

NOVEL BIOMARKERS TO IMPROVE PATIENT OUTCOME

HCC patients are commonly stratified based on their liver function capacity as assessed by Child-Pugh score, which overall seems to be a better predictor for treatment outcome than the underlying etiology of HCC^[70,73]. Biomarkers that could predict treatment response are therefore urgently needed, esp. for targeted therapies and immunotherapy approaches^[74].

In a recent biomarker analysis of the sorafenib phase 3 STORM trial (BIOSTORM), none of the biomarkers related to angiogenesis or cell proliferation or other molecular markers like gene signatures or mutations could predict a treatment benefit or recurrence-free survival (RFS). Only p-ERK and microvascular invasion were associated with poor RFS. This study proposed a new 146-gene signature that identified about 30% of patients which benefit from sorafenib treatment. Interestingly, those patients

Table 3 Clinical trials with chimeric antigen receptor T cells cells in hepatocellular carcinoma

NCT	Antigen	Phase	Patients	Sponsor	Status	Comments
NCT02715362	GPC3	I/II	30	Company	Recruiting	HAI
NCT03672305	c-Met/PD-L1	I	50	Academic	Not yet recruiting	IV
NCT02723942	GPC3	I/II	60	Academic	Completed	
NCT03198546	GPC3	I	30	Academic	Recruiting	
NCT02395250	GPC3	I	13	Academic	Completed	[58]
NCT03349255	AFP	I	18	Company	Recruiting	IV vs HAI
NCT03130712	GPC3	I/II	10	Company	Recruiting	IT
NCT03084380	GPC3	I/II	20	Academic	Not yet recruiting	Combination with TACE
NCT02905188 ¹	GPC3	I	14	Academic	Not yet recruiting	
NCT03302403	GPC3	I	48	Academic	Not yet recruiting	
NCT03146234	GPC3	I	20	Academic	Recruiting	
NCT01935843	Her2	I/II	10	Academic	Unknown	
NCT02959151	GPC3	I/II	20	Company	Unknown	
NCT02587689	MUC1	I/II	20	Company	Unknown	
NCT03013712	EpCAM	I/II	60	Academic	Recruiting	

¹Location of study is United States, all other trials are conducted in China. HAI: Hepatic artery infusion; IT: Intratumoral injection; IV: Intravenous injection; TACE: Transarterial chemoembolization.

were also enriched in CD4+ T and B cells, NK cells and were associated with signature of poor response to immune checkpoint inhibitors^[75].

Analysis of the regorafenib phase 3 study in HCC (RESORCE), also recently identified a plasma protein expression profile [angiopoietin 1, cystatin B, oxidized low density lipoprotein (LDL) receptor 1, latency associated peptide of transforming growth factor β (TGF- β) and macrophage inflammatory protein 1 α (MIP1 α)] and 9 plasma miRNAs that were associated with increased overall survival and time to progression. The proposed soluble plasma protein biomarkers are also known to play a role in inflammation and HCC pathogenesis. Interestingly, none of these predictive biomarkers was so far shown to have prognostic relevance^[76].

Overall, the identification and validation of biomarkers in HCC was previously limited by the availability of tissue specimens as international guidelines did not mandate a biopsy sample for diagnostic purposes. Recently, guidelines from EASL recommend taking tissue and liquid biopsies from HCC patients participating in clinical studies which could improve this situation^[77]. Biomarker analyses in HCC are often limited by small sample size in respective subgroup analyses due to the diverse etiologic backgrounds like viral hepatitis, NASH/NAFLD, cirrhosis status etc. which all significantly impact on the underlying chronic inflammation or have direct influence on oncogenic pathways.

Numerous new diagnostic and prognostic biomarkers like GPC-3 or c-Met have been proposed recently and were also translated into CAR-T cell based therapy approaches (see above) but no clear predictive biomarker for either targeted therapies or for immune checkpoint inhibitors is available so far^[78,79].

Liquid biopsies are capable of detecting genetic and epigenetic alterations as well as expression patterns of DNA, RNA and miRNA from circulating tumor cells, cell-free nucleic acids or from exosomes. Success rate of these technologies is still variable depending on tumor size and stage but with further technological advances, *e.g.*, on next generation sequencing from cell-free DNA, it is rapidly maturing to clinical applicability^[80-82].

Assessment of metabolic pathways including proteomics and glycomics could further contribute to biomarker development although current approaches, *e.g.*, detection of CD44v9^[83] or Hippocalcin-like 1 (HPCAL1)^[84], are used for diagnostic or prognostic settings or to predict disease recurrence and have not been linked to treatment responses.

NEW PRECLINICAL MODELS TO IMPROVE HCC THERAPY

The successful development of novel drugs is largely dependent on the availability of suitable and predictive preclinical models. Besides the specific biochemical (cell-free)

inhibition of a distinct target, novel compounds need to prove their potency in various *in vitro* and *in vivo* model systems before entering human clinical trials.

In the past, high throughput screening was performed on 2D cultures using immortalized cell lines. Although this approach allowed screening large numbers of cell lines, it lacks the complex interaction of different cell types and matrix structures within real tissue. Consequently, more complex 3D culture systems were established, also from primary human cancer samples, that also contain components of extracellular matrix and additional cell types as fibroblasts^[85,86]. Recently, spheroids and organoids that mimic organ structure and aggressiveness of human HCC have been established as tools for drug sensitivity screening^[87,88]. To reflect the genetic heterogeneity of human cancers, mixtures of barcoded tumor cell lines can be subjected to high throughput screening. This technology secures homogeneous drug exposure to genetically different cell types at the same time and was shown to identify responders and non-responders to specific treatments as well as to create new biomarker hypotheses^[89].

Precision-cut liver slices represent an interesting *ex vivo* model system for drug development. Complex tissue architecture is preserved and the model allows to investigate different pathophysiologic conditions and drug testing on primary human tissue samples^[90,91].

Animal models, however, still represent standard models for early drug development approaches. As HCC usually develops on the background of chronic underlying liver diseases associated with chronic inflammation, viral infection or fibrotic remodeling, and clear oncogenic driver mutations have not been identified yet, finding suitable and appropriate models still remains an urgent task. An ideal model would therefore in parallel describe the underlying liver disease and tumor development. The increasing role of immunotherapies in HCC also urgently warrants the development of respective immunocompetent models (Table 4)^[92].

The subcutaneous implantation of HCC cell lines was extensively used in the past and still has value when using primary cell lines or tissue explants (patient-derived xenograft models). These models are relatively easy to handle and provide an easy readout of tumor growth by caliper measurement. Orthotopic implantation reflects the primary site of tumor formation and crossplay with liver matrix and cells but requires surgical expertise and more advanced imaging technologies to monitor tumor growth. Both systems need immunodeficient mice unless syngeneic murine tumors are used. This limits the application of those models for studying immunotherapy approaches.

As human HCC can also develop upon chronic exposure to toxic agents, chemical induction in mice using diethylnitrosamine (DEN) has been established as a standard method but is limited by high variability of tumor formation and time to develop tumors^[93]. Yet, chemical models can easily be combined with other approaches (*e.g.*, fibrosis induction, NASH models, alcohol) and thus provide an option for rapid evaluation of novel drugs under distinct pathophysiologic conditions^[94-97].

Genetically engineered mice (GEM) are useful tools to study the contribution and effects of individual genes on HCC pathogenesis. They are technically more demanding and may have a longer latency period than other models. With the option of targeted knock-in and knock-out systems and combinations of those approaches, distinct molecular backgrounds can be analyzed. As today no clear single oncogenic driver for HCC development has been identified, several GEM models have been established as useful tools, *e.g.*, p53-deficient^[98] mice or mice overexpressing MYC^[99] or WNT pathway components^[100,101]. The option to study HBV and HCV transgenic mice is of special interest. We have shown previously that dependent on the host genetic background, ER stress pathways can be activated that are known to lead to cellular stress and chronic inflammation and that are involved in fibrogenesis and ultimately also HCC pathogenesis^[102,103].

As outlined above, HCC commonly develops on the basis of an underlying chronic liver disease. Therefore, specific liver disease models are of very high relevance to study HCC pathogenesis and explore new therapeutic options^[92]. Several small animal models for the development of liver fibrosis and cirrhosis are currently established. Application of carbon tetrachloride (CCl₄) or thioacetamide (in combination with ethanol)^[104] leads to rapid fibrosis development and acute inflammation^[105]. While easy to handle, these models can be combined with GEM or orthotopic transplantation models and provide a good tool to study tumor cells with a fibrotic micro-environment.

Although less clear in its pathogenesis for HCC, the increasing prevalence of metabolic liver diseases associated with diabetes mellitus, adipositas, hyperlipidemia, hypertriglyceridemia and metabolic syndrome puts models for NAFLD/NASH in the focus of today's research models^[106-108]. NAFLD and NASH can easily be induced by dietary models, *e.g.*, using a high-fat (HFD), high-cholesterol (HCD), high-fructose or

Table 4 Available techniques for induction of hepatocellular carcinoma in relation to temporal and technical aspects as well as major advantages and disadvantages (summarized from^[92])

Method and specification	Time to HCCshort (+) to long (+++)	Technical effortslow (+) to high (+++)	Major “Pros” (+) vs “Contras” (-)
Chemotoxic agents linked models			
Diethylnitrosamine	++	+	(+) good combination options with other methods (-) time to HCC not easily predictable
9,10-dimethyl-1,2-benzanthracene			
Direct implantation of tumor cells or tissue			
Heterotopic/orthotopic	+	+ / ++	(+) heterotopic xenografts are often and easily done (+) syngeneic orthotopic models better reflect the natural liver microenvironment (-) xenografts need immunocompromised mice (-) orthotopic tumor implants need surgical and imaging experience
Syngeneic/xenografts			
Genetically engineered mouse models			
Mouse embryo manipulation	++ / +++	+++	(+) hepatocarcinogenesis can be analyzed stepwise (-) effects of manipulated gene(s) could have heterogeneous latency and genetic penetrance
Cre-Lox recombination			
Hydrodynamic injection			
CRISPR-Cas9			
Humanized mouse models			
Immunologically humanized mice	+++	+++	(+) immunotherapeutical issues can be studied based on human cell lines in mice (-) establishment difficult due to engraftment failure and development of stable stem cell-derived hepatocytes
Genetically humanized mice			

HCC: Hepatocellular carcinoma.

methionine and choline-deficient (MCD) diets or combinations of those classical inducers^[109]. Yet, dietary models are often limited by not completely following the human course of disease, *e.g.*, lacking HCC formation for high-fat diet or lacking obesity for the MCD diet. Therefore, models have been further refined by combining the dietary stimulus with distinct genetic models like PTEN-deficient mice, MC4R (melanocortin 4 receptor) or ALR (augmenter of liver regeneration) knockouts that reliably lead to HCC formation in 60% to 100% after approximately one year^[110]. Recently, it was shown that HFD and HCD triggers liver cancer formation in an ApoE/LDL-receptor double knockout mouse, linking metabolic stress and atherosclerosis to HCC formation^[111].

CONCLUSION

Integrative and comprehensive molecular and genomic analyses could classify hepatocellular carcinoma on the basis of landscapes of genetic and molecular signatures (Tables 1 and 2) which could then lead to the identification of predictive biomarkers for novel treatment options and then impact HCC trial design and patient outcome. Consequently, all HCCs should be biopsied and specimen should be intensively investigated applying “omics”-technologies for real precision medicine approaches. Furthermore, the biological roles of the identified driver genes in HCC must be analyzed in the deeper integration of inter- and intratumoral, interpatient, and inter-ethnic tumor heterogeneity in more detail. Deeper knowledge about those drivers is urgently needed, as the underlying pathogenesis of HCC is complex and currently shifting from chronic viral infections to more metabolically driven tumorigenesis as seen in NASH. As inflammation seems to be a common ground for HCC development, it is not surprising that immune checkpoint inhibitors are moving into first line therapy setting and it is expected that this compound class will also

significantly shift the therapeutic landscape in HCC soon. Therefore, tumor models with complex genetically engineering are an essential drug development and technology transfer tool closing the gap between *in vitro* experiments and intensive clinical trials in future (Table 4). Finally, artificial intelligence and machine learning could essentially help to analyze, to classify and to interpret the dramatically increasing and high-dimensional amount of transcriptomic, genomic, epigenomic, metabolic, proteomic and imaging data in HCC^[112-114]. The desirable aims of such approaches will be to (1) identify cancer drug targets, (2) predict anticancer sensitivity, toxicity and cancer resistance, and (3) give robust recommendations for therapeutic strategies in the future^[115,116].

Overall, the better understanding of the molecular pathogenesis of HCC allows for more stringent patient selection criteria in biomarker-driven studies that can improve patient outcome.

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