REVIEW

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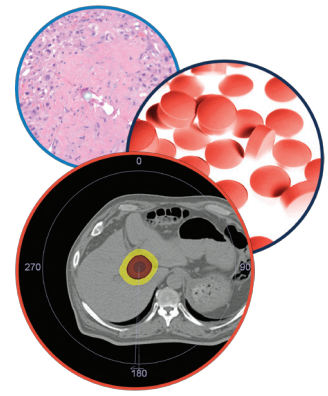

Hepatic Oncology

Liver cancer oncogenomics: opportunities and dilemmas for clinical applications

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Practice Points

- Primary liver cancer is characterized by complex molecular heterogeneity, stressing the difficulty of optimal clinical management.
- Therapeutic progress in liver cancer is hampered by genetic diversity and a lack of clear oncogene addiction. However, several recurrent somatic mutations have been identified by deep sequencing over the last few years.
- The JAK/STAT pathway, as a significant mediator of the immune response in the inflammatory microenvironment, may represent a drugable opportunity for a subset of primary liver cancer patients.
- Other potential targets involve receptor tyrosine kinases such as the MET oncogene in patients with high MET expression.
- Epigenetics and chromatin remodeling factors represent novel target options.
- Given the limited therapeutic options and poor outcome of patients with primary liver cancer, next-generation sequencing facilitates the promise of personalized therapeutic decision-making by enabling the direct targeting of unique genomic alterations, which drive preneoplastic lesion to advanced tumor stage, metastasis and recurrent disease for the individual patient.

SUMMARY Primary liver cancers are among the most rapidly evolving malignant tumors worldwide. An underlying chronic inflammatory liver disease, which precedes liver cancer development for several decades and frequently creates a pro-oncogenic microenvironment, impairs progress in therapeutic approaches. Molecular heterogeneity of liver cancer is potentiated by a crosstalk between epithelial tumor and stromal cells that complicate translational efforts to unravel molecular mechanisms of hepatocarcinogenesis with a drugable intend. Next-generation sequencing has greatly advanced our understanding of cancer development. With regards to liver cancer, the unprecedented coverage of next-generation sequencing has created a detailed map of genetic alterations and identified key somatic changes such as *CTNNB1* and *TP53* as well as several previously unrecognized recurrent disease-causing alterations that could contribute to new therapeutic approaches. Importantly, these investigations indicate that a classical oncogene addiction cannot be assumed for primary liver cancer. Therefore, hepatocarcinogenesis can be considered a paradigm suitable for individualized medicine.

KEYWORDS
• cholangiocarcnoma • hepatocellular carcinoma • individualized medicine • liver cancer • next-generation sequencing • oncogenomics

Background

The field of 'omics' is a relatively new scientific discipline. As technology advances, the cost of genome characterization from conventional sequencing to next-generation approaches and the scale and scope of inquiry has successively broadened from unprecedented biological questions to include clinical application. Next-generation sequencing (NGS) is complementing

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individualized medicine on multiple levels, which include prevention, risk prediction, diagnostics, therapeutics and many more aspects. This review explores the current state of somatic variation with a focus on the results of recent NGS studies in hepatocarcinogenesis.

Over the last decade a detailed map of the structural variation in the human cancer genome has been generated. This map delineates how tumors may develop as the consequence of, for example, intragenic mutations, in as little as roughly 140 genes, which belong to 12 distinct signaling pathways surrounding three core cellular processes: cell fate, cell survival and genome maintenance. Alterations in these processes are major genetic drivers to promote tumorigenesis in the majority of human cancers [1].

Primary liver cancers, that is, hepatocellular carcinoma (HCC) and cholangiocarcinoma (CCA), are a serious global health problem with more than 1 million cases diagnosed annually [2]. These malignancies are the second-most common cause of death after lung cancer and, besides melanoma and pancreatic carcinoma, among the few solid malignancies with increasing incidence and mortality rates worldwide. HCC is the fifth-most common cancer in men and ranks seventh in women worldwide, accounting for at least 700,000 deaths annually [3]. Etiological factors include nonalcoholic fatty liver disease and other metabolic disorders that have become particularly relevant in Western countries due to an increase in prevalence and a high number of HCCs without underlying cirrhosis [4]. The major etiologic agents, such as infection with either hepatitis B (HBV) or C viruses (HCV), as well as alcohol abuse, are the main causes for chronic liver disease, progression to cirrhosis and, ultimately, HCC development. During the past decade, molecular mechanisms of chronic liver diseases have been identified that are associated with increased risk of HCC as well as several cellular alterations that precede HCC development [5,6]. In contrast to HCC, a clear picture of the underlying causes resulting in CCA is still debated [7]. A background of chronic liver inflammation, for example, primary sclerosing cholangitis and cholestasis, are linked to increased risk of developing CCA. Other risk factors, similarly to HCC, include hepatitis infection, alcohol consumption, and obesity and/or diabetes. Regional specific hazards unique to CCA include parasitic liver infestation, which is endemic predominantly in north-east Thailand.

Research into the molecular pathogenesis of liver cancer is currently focused on the interrelationship of abnormal genomics, epigenomics, proteomics and metabolomics as well as integrating this information into a causative map of the downstream alterations in molecular signaling pathways with the potential for targeted therapies. Unfortunately, owing to lack of initiatives, the pursuit of genomics in clinical decision-making and advancement of patients' outcome has not reached hepatic oncology. Following the approval of sorafenib for HCC [8], several Phase III trials in liver cancer have failed to meet their primary end point of survival [9]. A significant drawback in design of these trials was the lack of molecular target stratification and acquisition of tissue for molecular analyses. This era of disappointing results in liver cancer therapeutics highlights the difficulty of clinical management in these malignancies and the urgency for novel therapeutic options. The primary goal of this review is to highlight the key molecular alterations, which may be implemented in future clinical trial design as markers for patient stratification and drug targeting.

Molecular hepatocarcinogenesis: from chronic hepatitis to liver cancer

Somatic variants and chromosomal aberrations in tumors are traditionally regarded as evidence of gene deregulation and genome instability, and may facilitate the discovery of crucial biomarkers/genes and identification of regulatory pathways that are perturbed in the disease [10]. Large genome-wide association studies recently identified liver-disease-specific susceptibility loci, including MICA on 6p21.33 (rs2596542) in HCC [11,12]. Most of these studies employed high-throughput microarray technology for single nucleotide polymorphism (SNP) genotyping and array-based comparative genomic hybridization. These technologies enable high-throughput analysis of DNA copy number variations (CNV) and yield comprehensive information applicable to determining the molecular pathogenesis of human liver cancer. A recent meta-analysis suggested that both intrahepatic CCA (iCCA) and HCC share common CNVs, including chromosomal gains (1q, 8q and 17q) and losses (4q, 8p, 13q and 17p), with high-level amplifications of 11q-13 (reviewed in [13]), indicating that iCCA and at least a subgroup of HCCs are closely related at the molecular level. Indeed, a close genomic similarity between iCCA and a subset of HCCs

with progenitor cell characteristics and poor outcome was shown in several recent studies [14–16]. Moreover, genomic [15,16] and genetic [17,18] analyses of the rare mixed HCC-CCA type closely show related alterations and shared molecular characteristics between iCCA and HCC tumors, which suggest that the acquisition of CCA-like transcriptomic traits plays a critical role in the heterogeneous progression of liver tumors.

Clinically, NGS has been proven powerful particularly in the detection of viral infection, for example, HCV in liver biopsies [19]. Deep sequencing has also been applied in a longitudinal analysis of the viral evolution following early viremia in four asymptomatic acute HCV infected patients where blood samples were collected over the initial 24 weeks [20]. Development of vaccines against, for example, HCV, requires an understanding of selective pressure on the viral population/genomes following infection. Thus, parameters such as: the founder strain that effectively infects the host/patient, and the primary infection, which either results in clearance or drives disease progression, cause chronic infection and lastly liver disease, and are important to efficiently control.

Spectrum of genetic alterations in liver cancer

The NGS of the last few years has increasingly been applied in cancer research, including liver cancer, and generated an integrative view on different molecular levels, that is, genomics, transcriptomics and epigenomics in carcinogenesis [10,21]. Since liver cancer often arises in the background of chronic liver disease and underlying liver cirrhosis, implementation of NGS is generally challenging. Thus, the cellular composition within a given tumor such as infiltrating immune cells, stromal compartment and fibrotic/connective tissue, significantly influences the genomic signature [22]. To completely understand the molecular mechanisms of hepatocarcinogenesis, the cellular complexity as well as the origin of malignant transformation [23], in other words hepatocytes, cholangiocytes and stem/progenitor cells, have to be appreciated and will likely either require additional sequence coverage or, preferably, selection and analysis at single-cell resolution.

• Hepatocellular carcinoma

Hepatocarcinogenesis likely resembles a branched multi-stage process that develops on the basis of sequential acquisition of molecular alterations. Dysplastic nodules (low-grade and high-grade) usually develop in cirrhotic livers and subsequently transform to early HCC that ultimately progress into advanced/progressed HCC. Although the different lesions are morphologically distinct, the molecular alterations driving this process are not clearly defined [24]. Prediction of which lesions are progressive, in other words, at risk for malignant transformation, is currently questioned and represents a cause for clinical concern. This lack of adequate information represents a major challenge for preventive strategies and therapeutic approaches in HCC. Although a study recently attributed a key oncogenic role to the activation of the MYC oncogene during conversion of dysplastic nodules to early HCC, a detailed molecular understanding of this process is lacking [25]. Acquisition of adequate samples and the analysis of the clonal evolution in liver cancer remain considerably difficult since these investigations require analyses of the full spectrum of lesions observed in the liver, ideally in large numbers and within the same patients. Further, current clinical guidelines recommend ultrasound follow-up of lesions <1 cm, which reduces the probability of obtaining early lesions [26]. The authors have recently performed an integrative transcriptome sequencing analysis of tumor-free surrounding liver (n = 7), low- (n = 4) and high-grade (n = 9) dysplastic lesions, early HCC (n = 5) and advance/progressed HCC (n = 3) from a total of eight HCC patients with cirrhosis due to chronic HBV infection [27]. Interestingly, the study determined that the molecular profiles of dysplastic and early lesions are relatively uniform followed by a sharp increase in heterogeneity at both the genetic and genomic level upon advancement into advanced/progressed HCC. The progressive accumulation of genetic alterations late in hepatocarcinogenesis resulted in a substantial deregulation of key oncogenic molecules such as TGFβ, MYC, PI3K and AKT as well as activation of prognostic adverse signaling pathways [27]. This was further underscored by the observation that genetic variants with impact on gene expression affected known oncogenic drivers such as *TGFβ1*, *NOTCH2*, *VCAM1*, *JAK1*, *IGF2*, *IGFBP5* and *MMP14*, which were only detected during late stages of the disease and may support a multi-hit theory in hepatocarcinogenesis. One somatic variant, which was detected in the *IGFALS* gene (hs16_1840768),

caused a progressive down-regulation of *IGFALS* from cirrhosis to advanced/progressed HCC. The identified aberration was predicted to have a protein-damaging effect that may potentially promote HCC by causing the activation IGF signaling. This indicates that *IGFALS*, which encodes a soluble serum protein binding the IGF, is a potential marker with applications in diagnostic and therapeutic interventions. Notably, alterations in *IGFALS* were previously recognized in advanced HCC [28].

Application of whole-genome or -exome sequencing has currently been limited to advanced HCC. As already delineated, chronic viral hepatitis (i.e., HBV and HCV) as well as alcohol consumption are predominant risk factors for HCC development, and are therefore the subject of recent investigations [3]. HCV infections are the dominant risk factors for HCC development in Japan. Consequently, several Japanese studies elucidated the impact of HCV on the development of liver cancer. The first whole-exome sequencing (WES) study of a Japanese male in 2011 identified a total of 88 validated somatic alterations, which included mutations in *TP53* and *AXIN1* [29]. More recently, WES was applied to ten HCV-related HCC cases and corresponding matched normal tissues [30]. Overall, the average rate of mutation per tumor was ~43 variants. Interestingly, only five genes (*CTNNB1*, *TP53*, *ARID2*, *DMXL1* and *NLRP1*) showed recurrent somatic mutations in more than two tumors. Validation of these alterations in a larger and etiologically more diverse cohort of HCC cases (HBV, HCV, mixed or non-viral background) showed that around 18.2% of individuals with HCV-associated HCC in the USA and Europe harbored genetic variants in the *ARID2* gene. Interestingly, the inactivating mutations in *ARID2* were further associated with alterations observed in *CTNNB1* but mutually exclusive with *TP53* mutations, which are known to be associated with HBV infection.

Another study applied WES to a mixed cohort of 27 viral-hepatitis-associated HCCs (25 HBV and two HCV), including two sets of multicentric tumors. Interestingly, no common somatic mutations were identified in the multicentric tumor pairs, suggesting the late acquisition of genomic complexity. Furthermore, comparison of their whole-genome substitution patterns suggested that these tumors developed on the basis of independent genetic branching [31]. Although etiological differences likely induce distinct somatic alterations, no significant differences in the number of somatic substitutions, indels and rearrangements could be revealed between HBV- and HCV-related HCCs [32]. Excessive alcohol consumption as well as the presence of multiple liver nodules possessed significant impact on the somatic substitution patterns. Overall, the average number of nonsynonymous mutations in this study was 75.9 per tumor and involved missense as well as nonsense mutations, short coding indels and splice-site mutations. In agreement with previous observations, mutations in *TP53, CTNNB1* and *EGFR* were the most frequent alterations in the advanced HCCs. Furthermore, as demonstrated by Li *et al.,* a high incidence was observed in mutations of chromatin regulators such as *ARID1A, ARID1B, ARID2* and *MLL3*, which comprised about half of all tumors. To date, the largest NGS study in liver included 88 cases of advanced HCCs mainly related to HBV infection [33]. This sizeable whole-genome sequencing (WGS) study confirmed the prominent role of *CTNNB1* (15.9%) and *TP53* (35.2%) mutations. With regards to signaling pathways, genes associated with the WNT/CTNNB1 and JAK/STAT pathways showed the highest incidence of genetic alteration (62.5 and 45.5%, respectively). In addition, the study functionally highlighted the importance of prevalent drugable mutations such as JAK1, which were observed in up to 9.1% of patients. Another recent NGS study analyzed the transcriptomes of ten matched HBV-infected livers and HCC cases, and identified a total of 1378 differentially expressed genes with functional enrichment of gene sets associated to cell growth, metabolism and inflammation, displaying a specific enrichment of genes related to chromosome location 8q21.3–24.3 [34].

In addition to somatic alterations several studies have also evaluated the significance of cancer-promoting HBV genome integration sites. Fujimoto *et al.* [31] identified, at a high frequency, recurrent integration sites at the *TERT* locus, which supports the observation that this event may confer a growth advantage in the early phase of HBV-related liver carcinogenesis. The importance of HBV viral integration at the *TERT* locus was subsequently validated in hepatocarcinogenesis [35]. NGS of 81 HBV-positive and seven HBV-negative HCCs, as well as their adjacent normal tissues, found that HBV integration increasingly occurred

during hepatocarcinogenesis and further led to the induction of both chromosomal instability and CNVs. Interestingly, Nault *et al.* recently applied Sanger sequencing to a Western cohort of 305 HCCs, which included cases related to HBV, HCV, alcohol and hemochromatosis as well as cirrhotic preneoplastic macronodules [36]. Despite the etiological differences, the study identified activating somatic mutations in the *TERT* promoter, which comprise the earliest and most frequent genetic targets in human preneoplastic liver lesions (25%) as well as HCC (59%), and further showed an association with activating *CTNNB1* mutations. Notably, given the location of these mutations within the promoter region, detection in studies focused on the coding region was not possible.

An integrated analysis of CNVs and genetic alterations by WES (24 HCC) as well as single nucleotide polymorphism arrays (125 HCC) identified 135 homozygous deletions and 994 somatic mutations in genes with predicted functional consequences [37]. The most common mutations were observed in *TP53* and *CTNNB1*. This study also detected an over-representation of G:C>T:A nucleotide transversions in non-cirrhotic livers and well-differentiated tumors, which support the perception that genotoxic stress may contribute to these transformation events [38]. Additionally, the authors identified previously unrecognized recurrent somatic mutations in *ARID1A*, *RPS6KA3*, *NFE2L2* and *IRF2*. Subsequent functional analyses attributed tumor suppressor properties to IRF2. Inactivation of IRF2 was exclusively found in HBV-related tumors associated with impaired TP53 function. Further, alterations in chromatin-modifying genes were observed in around 25% of tumors related to alcohol consumption. Cleary *et al.* recently analyzed a representative western collective of 87 HCC cases (38 HBV, 19 HCV, ten alcohol, three hemochromatosis) [39]. In agreement with the above-mentioned studies, an average of 45 predicted protein-damaging mutations (range 2–381) was observed, which involved frequent *TP53* (18%) and *CTNNB1* (10%) as well as the chromatin regulators (20%).

Two recent NGS studies investigated the role of RNA editing in hepatocarcinogenesis [40,41]. Chen *et al.* thoroughly investigated three paired non-tumor and tumor specimens, and demonstrated that adenosine-to-inosine (A→I) RNA editing of *AZIN1* is significantly increased in HCC versus normal liver specimens [41]. RNA editing of *AZIN1* was specifically exerted by ADAR1 and resulted in a serine-to-glycine substitution at residue 367 of AZIN1, causing conformational changes leading to increased tumor-initiating potential as well as more aggressive phenotypes [41]. In continuation of this work the group showed that HCC patients with ADAR1 overexpression and ADAR2 downregulation displayed an increased risk for liver cirrhosis and postoperative recurrence as well as overall poor outcome [40]. The differential regulation of ADAR1 and ADAR2 in HCC altered gene-specific editing activities and was reflected by the hyper-editing of *FLNB* and the hypo-editing of *COPA*. *In vitro* and *in vivo* functional analyses subsequently confirmed the oncogenic and tumor-suppressive properties of ADAR1 and ADAR2 respectively. Overall these investigations confirm that RNA editing may play an important role in promoting hepatocarcinogenesis.

• Hepatocellular adenoma

Hepatocellular adenomas (HCA) are a rare subtype of primary human liver tumors. HCA are usually hormone-sensitive, monoclonal benign liver lesions that are associated with the use of estrogen-rich contraceptives or androgen-containing steroid anabolics, which develop in cirrhotic livers and rarely undergo malignant transformation. HCA are classified into four morphological and molecular subtypes [42]: HNF1α-mutated HCAs (H-HCA, 30 to 40% of all adenomas), characterized by downregulation of LFABP and recurrent loss of heterozygosity at chromosome 12q leading to biallelic somatic mutations in the *HNF1A* gene; telangiectatic/inflammatory adenomas (IHCA) (40% to 50% of all adenomas) with activating mutations in *IL6ST, GNAS* or *STAT3*. IHCA frequently show activation of the JAK/STAT pathway and around 50% harbor mutations in *CTNNB1*; β-catenin-mutated adenomas (b-HCA, 10 to 15% of all adenomas) characterized by activating mutations of *CTNNB1,* predominantly in exon 3, that are mutually exclusive with *HNF1A* mutations. The b-HCA subtype has the highest potential of malignant transformation in around 5% of the lesions; unclassified adenomas (u-HCA; 10% of all adenomas) with no specific morphological or immunophenotypical pattern. A recent study by Pilati *et al.* provided the only NGS analysis so far, of 35 HCA [43]. In comparison to other tumors the average rate of protein-damaging

mutations was relatively low (7.5 per tumor) and recurrent alterations were only observed in four genes (*CTNNB1*, *IL6ST, HNF1A* and *FRK*). The presence of these previously unrecognized somatic mutations in *FRK*, as well as *JAK1* in b-IHCA and IHCA, were of particular functional relevance and potentially drugable. Further, integrative copy-number and methylation profiling accurately recapitulated the different clinical subtypes. Most interestingly, the study demonstrated that in addition to *CTNNB1* mutations that occur relatively early, *TERT* promoter mutations are frequently observed in later stages of the adenoma-to-carcinoma transition, potentially as a required second hit. These investigations indicate that, in addition to *CTNNB1* mutations, surveillance of HCA should be extended to *TERT* promotor mutations [44].

• Intrahepatic cholangiocarcinoma

CCA is an orphan cancer type with limited understanding of its genetic and genomic pathogenesis [45]. iCCA is a malignancy of the interlobular bile ducts, whereas tumors designated perihilar are generally considered extrahepatic (pCCA) and originate in the main hepatic ducts or at the bifurcation of the common biliary tract. Tumors which emerge in the extrahepatic bile duct distal to the liver and advance towards the gallbladder, are designated dCCA and are embryonically distinct from iCCA. The majority of dCCA cases are associated with a concomitant inflammatory bowel disease and/or primary sclerosing cholangitis, while iCCA share similar risk factors such as HCC. Whereas overall cancer mortality has declined, the mortality rate and incidence of CCA is rising [46]. In Europe, the epidemiological trend is mixed [47]; however, iCCA stands out and in Germany, for example, the disease has more than tripled in the past decade irrespective of a preconditioned inflammatory disease, that is, primary sclerosing cholangitis [48]. This malignancy is characterized by clinical and pathobiological heterogeneity, which complicate treatment as well as assessment of efficacy of therapeutic agents. Tumors often cause clinically few symptoms and are therefore diagnosed at a late stage, for example because of obstructed bile drainage causing jaundice. Currently there are no approved systemic therapies for CCA [49] and at diagnosis about 70% of tumors are unresectable. As such, the 5-year survival rate for these patients is 0–10% [50,51]. Standard chemotherapy for CCA is palliative and includes the combination of gemcitabine backbone with a platin-based drug, for example, oxaliplatin (GEMOX) or cisplatin (GemCis) [52]. Inadequate response to therapy is likely caused by hepatotoxicity and genomic complexity of the primary tumor, which may be linked to malignant and recurrent disease.

Although genomic studies of iCCA are limited (reviewed in [13,49,53]), progress has been made and several current omics-based studies detail various aspects of the CCA genome landscape, for example, integrative transcriptomics [14,54] and epigenomics [55,56]. Whereas recent interest in understanding the molecular complexity of CCA has been evident, the major obstacles for a path forward include access to well-preserved and annotated cohorts. Mainly due to the anatomical complexity of this malignancy (iCCA, pCCA and dCCA), heterogeneity of the cohorts studied may be considered as a confounding factor that should be addressed according to the current CCA International Liver Cancer guidelines [45].

NGS was first used to describe the genetic variation of liver-fluke-related CCA [57]. *Opisthorchis viverrini* is a trematoda, endemic in Thailand, Laos and Malaysia, associated with a 100-fold increase in the development of CCA and constituting a major public health concern in these areas. The authors performed WES of eight *O. viverrini*-related tumors and matched normal tissues, and validated a total 206 somatic mutations in 187 genes using Sanger sequencing. The average number of nonsynonymous variants was 26 per tumor, ranging from 19 to 34. The predominant somatic substitution was C:G>T:A transitions. Frequent somatic mutations were found in key genes such as *TP53* (44.4%), *KRAS* (16.7%) and *SMAD4* (16.7%). Additionally, alteration in ten previously unrecognized genes included inactivating mutations in *MLL3*, *ROBO2*, *RNF43* and *PEG3* as well as activating mutations in the *GNAS* oncogene. As similarly observed for HCC, novel mutations are centered on chromatin remodeling and genome stability, thus underlining the importance of genes involved in histone modification for liver cancers other than HCC. Exome-sequencing of 15 non-liver-fluke-associated CCA cases that included ten iCCAs and five extrahepatic cases, determined a distinct genetic pattern related to this regional-specific risk factor [58]. The study, which included

a prevalence screen of 108 CCAs associated with fluke infestation and 101 cases of non-fluke-related etiology, unfortunately included tumors from mixed anatomical origin. This complicates the decision of the mutational rate specific to iCCA, since some genetic variants such as *KRAS* are more prevalent in pCCA [14]. The mean somatic mutational rate of non-fluke-infested CCA was 16 per tumor (range one to 62), a significant lower mutational burden compared with fluke-infested tumors, which may in part explain the elevated disease risk caused by the parasitic infection. Importantly, Chan-on *et al.* identified a significant degree of variation in chromatin modulators such as *BAP1* (10%) and *ARID1A* (10%) as well as confirmed the presence of key somatic mutations in the genes encoding *IDH1* and *IDH2*. Beside these genetic variants, recurrent mutations were identified, that is, *AGPAT6, ATP10A, BRPF3, CCT8L2, GPR112, HMCN1* and *LRRIQ1*, which are all involved in diverse functional processes and as such may represent 'subclonal drivers'. Analysis of an additional 32 iCCA cases with a mean of 39 somatic mutations per genome (ranging from 13 to 300), identified inactivating mutations in multiple chromatin remodeling factors with a high prevalence in *BAP1* (25%), *ARID1A* (19%) and *PBRM1* (17%) [59]. Interestingly, the authors found that 47% of iCCA cases have somatic alterations in at least one chromatin-modifying gene, which was not mutually exclusive and suggests ongoing accumulative epigenetic changes as well as worse overall survival. As such, resected patients with *IDH* mutations were found to have a significantly reduced 3-year survival rate of 33%, compared with 81% for patients with wild type *IDH* status. Importantly, mutations in chromatin-remodeling factors, as well as *IDH1* and *IDH2,* may result in altered sensitivity of these tumors to drug targeting, for example HDAC inhibitors or demethylating agents. Recently, Gao and colleagues sequenced the exomes of seven Chinese iCCA patients and their surrounding non-tumoral tissue to detect somatic alterations [60]. Interestingly, these patients were all selected as treatment naïve and diagnosed without any background liver or biliary diseases, ensuring that there would be no clonal selection of the driving aberrations caused by prior treatment pressure. The study found a range of seven to 192 mutations per tumor, which is comparable to other studies; however, they observed a prevalence of transversions, compared with transitions, at a ratio of 3.7:1, including a predominant targeting of G/C nucleotides. Interestingly, a higher frequency of transversions was also recently described in HCC [37,61], suggesting a common origin of at least a subset of iCCAs and HCCs. A key finding in this study, which included a screen of 124 iCCA cases, was the prevalence of activating somatic mutations in *PTPN3* (41%), a gene shown to promote cell proliferation and migration, which correlated with overexpression of PTPN3 and tumor recurrence [60]. The degree of recurrent alterations detected in nine members (*PTPRB, PTPRQ, PTPRS, PTPRZ1, SBF1, SBF2, MTMR3* and *EYA1*) of the PTP family further increased to 51.6%, with at least one gene mutated per sample, making the PTP family the most commonly targeted pathway in iCCA. Of interest, the two largest cohorts utilized for patient stratification both demonstrate a predominant deregulation in receptor tyrosine kinase networks [14,54]. Recently, Morris *et al.* found frequent deletions of *PTPRS* in head and neck cancer, suggesting an activation of the EGFR and PI3K pathways [62]. Notably, alterations in *PTPN3* have not been confirmed in any other genomic profiling studies, indicating that this may be an ethnic-specific aberration in the Chinese population.

A recent target-specific, exome-sequencing hybridization-capture approach evaluated 182 cancer-related genes in 28 formalin-fixed, paraffin-embedded iCCA cases, showing that up to two-thirds of tumors harbor actionable genomic aberrations with the potential to influence the treatment decisions for these patients [63]. The study also introduced two novel gene fusions involving tyrosine kinases FGFR2 and NTRK1. Several FGFR2 gene fusion products have been reported in CCA, including *FGFR2-BICC1* (2/4) [64], *FGFR2-KIAA1598* (1/28) [63], *FGFR2-TACC3* (1/28) [63], *FGFR2-AHCYL1* (7/66) [65] and *FGFR2-MGEA5* (1/6) [66]. Interestingly, Borad *et al.* identified FGFR2 gene fusions in 3/6 iCCA cases. This study is, to date, the only to include both transcriptome and WGS analyses of tumors from the same patients. Arai *et al.* concluded from a cohort of more than 100 CCAs that the *FGFR2* rearrangements only occur in iCCAs (9/66, 13.6%) [65]. Expression of FGFR2 leads to the activation of the MAPK pathway. These studies stress the urgent need for tailored clinical trials in CCA to evaluate

the efficacy of FGFR inhibitors such as ponatinib or ragorafenib.

New durgable tragets on the horizon

The landscape of genetic alterations in liver cancer is relatively broad, and ranges depending on their cellular origin (HCC: 41; HCA 7.5; iCCA: 27). Thus, the genetic heterogeneity of a given liver tumor involves a complex interaction of multiple distinct mutations, which ultimately drives malignant transformation [29–33,37]. Furthermore, the most frequent genetic alterations observed in liver cancer are detected in *TP53*, *CTNNB1* and cycle-related genes that include about half of all tumors (Table 1). Unfortunately, due to the essential functions of these genes for normal cellular processes and pleiotropic ways of activation, these observations are extremely difficult to translate into therapeutic approaches [32,67]. So far, no clear oncogenic addiction could be demonstrated in the different studies, which significantly hampers the development of novel therapies. As already mentioned, the impaired liver function additionally limits the application of classical chemotherapies. However, there are common mutations in several disrupted signaling pathways centering on epigenetics and inflammation, as well as classical oncogenes that could be targeted and are outlined below.

• Epigenetic modulation

Maintaining the integrity of the epigenome is a key component of organ homeostasis. Substantial evidence further suggests that disruption of epigenetic regulation is one of the fundamental mechanisms underlying many human diseases, including cancer [68,69]. This landscape of epigenetic alterations adds further complexity to the molecular pathogenesis of solid tumors. Epigenetics are highly influenced by and responsive to the tumor microenvironment. Therefore, changes in the epigenome are believed to be early events in carcinogenesis preceding allelic imbalances and ultimately lead to cancer progression [70]. Results of the recent NGS studies underlined the prominent role of epigenetic modifications for the development of liver cancer, in particular actionable somatic mutations in both *IDH1* and *IDH2* in iCCA. The results further highlight the potential of targeting epigenetic mechanisms in novel therapeutic strategies for liver cancer. A novel occurring target common to both HCC and iCCA that the recent NGS studies have highlighted includes frequent alterations in chromatin remodeling factors.

• Chromatin modifiers

Chromatin remodeling involves several factors (e.g., nucleosome remodelers and histone modifiers) and can actively impair gene expression; for example, by modification of histone tails such as phosphorylation at serine residues, methylation/acetylation of arginine, methylation (mono, di and tri), ubiquitination and sumoylation [71]. Overall, 16 to 24% of HCCs and 7.5 to 25% of iCCAs showed genetic alterations in pathways related to chromatin regulation (e.g., *ARID1A, ARID2, BAP1, MLL* and *PBRM1*), suggesting a causative association with hepatocyte transformation and highlighting recent evidence for the key role of epigenetics in hepatocarcinogenesis [72]. HDACs are important mediators of epigenetic transcriptional regulation. Several HDAC inhibitors have been tested in early clinical and preclinical investigations in liver cancer [73]. A recent study further indicates that the combination of sorafenib with the HDAC inhibitor panobinostat shows high anti-tumor efficacy in preclinical HCC models. Other interesting new targets are the SWI/SNF chromatin-remodeling complexes. Given the high incidence of genetic alterations in associated genes such as *ARID1A, ARID2, ARID4* in liver cancer (up to 36%), modulation of the pathway might be particularly promising [74]. The link between genetic alterations in *MLL* genes (myeloid/lymphoid or mixed-lineage leukemia, 2% of mutations) and the MET proto-oncogene further underlines the potential of epigenetic interventions in liver cancer [75].

• Aberrant regulatory non-coding RNAs

The deregulation of small regulatory RNAs, in particular microRNAs, with subsequent alterations of target gene expression, has been linked to the pathogenesis of most chronic liver diseases as well as hepatocarcinogenesis [76]. In this context, the identification of specific small RNAs with tumor-suppressive or oncogenic functions has greatly advanced our understanding of liver cancer development, and distinct expression profiles of microRNAs are significantly associated with liver cancer initiation, propagation and progression [77]. Emerging evidence further indicates that certain microRNAs directly contribute to cell proliferation, apoptosis and

Table 1. Key somatic variants in liver cancer.

Target genes	iCCA (%)	HCC (%)
Telomere maintenance		
TERT promoter	nr	20–60
WNT/β-Catenin		
CTNNB1, AXIN1/2, APC	0	2–35
Cell cycle	**4–36**	**4–35**
TP53	7–36	18–35
CKN2A/B, CDKs, RB1, ATM	4–7	4–12
IRF2	0	0–5
Apoptosis		
TNFRSF10A/B, TRADD, CASP, XIAP, MCL1[†]	0–21	8–20
Chromatin remodeling factors		
ARID1A	10–36	10–16
ARID2	nr	2–18
BAP1	11–20	nr
MLLs	0–17	1–8
PBRM1	0–13	0–3.5
PI3K/RAS pathway		
KRAS	0–20	0–1.5
NRAS	7–17	nr
HRAS	nr	0–1
RPS6KA3	nr	0–9
PIK3CA, PIK3C2G	4–17	0–2
PTEN	7–11	0–2
TSC1	0–4	0–1
Epigenetics		
IDH1, IDH2, IDH3A	0–35	0–1
Protein tyrosine phosphatase		
PTPN3 etc.	41–52	nr
FGF pathway		
FGF19	nr	4–15
FGFR2	0–14	0–2
FGFR2 fusion genes[‡]	~50	nr
JAK/STAT pathway	**0**[§]	**2–26**
JAK1	nr	0–9
IL6R	nr	0–26
IL6ST	nr	2–3
Oxidative stress response		
NRF2	nr	0–8
KEAP1	nr	0–6
TGFβ/SMAD pathway		
TGFβ, TGFβR1+2	0–4	0–1
SMADs	0–17[¶]	0–1

Somatic mutation not reported, nr.
Pathway with prevalence of somatic mutations in both HCC and iCCA is highlighted in bold.
[†]Gene amplification found in iCCA.
[‡]FGFR2 gene fusions with BICC1, KIAA1598, AHCYL1 and MGEA5 in iCCA.
[§]No somatic variants were reported in iCCA, however, the pathway is commonly activated in more than 50% of iCCA cases.
[¶]SMAD4 mutations reported in iCCA associated with liver fluke infestation.
HCC: Hepatocellular carcinoma; iCCA: Intrahepatic cholangiocarcinoma.

metastasis of HCC as well as correlate with several clinicopathological features [78]. Loss of expression of miR-122, one of the most prevalent microRNAs in the liver, is associated with poor prognosis and favors a metastatic potential through increased cell migration and invasion that results in loss of hepatic differentiation [79]. Furthermore, several microRNAs have been associated with key molecules in hepatocarcinogenesis such as WNT/β-catenin, MYC and TGFβ, and could therefore be a target for microRNA-based therapeutic strategies [80]. Although global analyses of small regulatory RNAs by next-generation sequencing is a promising application that provides unprecedented read depths, studies so far are limited to a few microRNA-centered investigations. One study that analyzed the differential expression of microRNAs in human normal liver, chronic hepatitis and HCC, identified nine microRNAs (miR-122, miR-99a, miR-101, miR-192, miR-199a/b-3p and several let-7 family members) accounting for ~88.2% of the 'miRNome' in human liver [81]. Further, decreased miR-199a/b-3p expression significantly correlated with survival of HCC patients. Moreover, targeting of miR-199a/b-3p, using adeno-associated virus 8, inhibited tumor growth via interacting with PAK4/Raf/MEK/ERK pathway. The clinical relevance of this microRNA was further confirmed by a recent study that also showed the inverse correlation between miR-199a-3p with mTOR and c-Met associated with a shorter time to recurrence after HCC resection [82]. Another global microRNA analysis in 104 HCC, 90 adjacent cirrhotic livers, 21 normal livers as well as in 35 HCC cell lines, detected a set of 12 microRNAs (including miR-21, miR-221/222, miR-34a, miR-519a, miR-93, miR-96, and let-7c) associated with malignant progression in liver cancer. Here miR-221/222 were the most upregulated microRNAs in HCC and identified to target the CDK inhibitor p27 as well as DNA damage-inducible transcript 4 (DDIT4), a modulator of mTOR pathway, to enhance cell growth *in vitro* [83,84]. Overall, great promise rests on these microRNA-based diagnostic and therapeutic approaches to improve the dismal outcome of liver cancer patients [85,86].

• The JAK/STAT pathway & inflammatory microenvironment

Binding of IL-6 to its receptor, gp130, triggers the heterodimerization with the Janus kinases (JAK1, JAK2 or TYK2), thus facilitating a signaling cascade and activation of STAT3, that is, the JAK/STAT, and the MAPK pathway.

The *JAK1* gene encodes a cytoplasmic tyrosine kinase that is associated with signal transduction from many cytokine receptors frequently associated with a variety of liver diseases [87]. Several mutated forms of JAK1 have been recognized in the context of hepatocarcinogenesis, often resulting in the upregulation of JAK1 with subsequent activation of the JAK/STAT pathway. The above-mentioned study by Kan *et al.* [33] detected activating mutations of *JAK1* (S703I and S729C) in almost 10% of the investigated HCCs. A recent study by Pilati *et al.* [43] additionally showed recurrent somatic mutations in JAK1 leading to STAT3 activation in HCAs, which highlights the role of this pathway as a potential preventive or therapeutic target in liver cancer. Consistently, targeting of the pathway in JAK1-mutated hepatoma cells significantly sensitized the cells to JAK1/2 inhibitor ruxolitinib.

Activation of the JAK/STAT signaling pathway has also been attributed to at least 50% of iCCA [88], however, none of the NGS studies to date have detected recurrent somatic mutations in this pathway (Table 1). Regardless, the JAK/STAT pathway represents an interesting and persuasive target option to take advantage of novel STAT3 or JAK1-JAK2 inhibitors such as AZD1480, or selective JAK1 inhibitors.

• Other novel target options

Several other actionable targets have been outlined in recent genomic studies that could potentially be useful for subclassification, and improved management and outcome of patients with liver cancer. Many of the new promising candidates involve the crosstalk between tumor and microenvironment, inflammatory cytokines and other factors activating cells, for example hepatic stellate cells, in the altered microenvironment (reviewed in [49]). These pathways include FGF and IGF receptors, Notch, Hippo, Hedgehog (Hh) and PLKs, as well as immunotherapies. Among the deregulated pathways in iCCA, *FGFR2* represent an exciting and novel actionable target that includes gene amplifications, somatic mutations and gene fusions. Gene fusions with *FGFR2* were detected in 13.6 to 50% of analyzed samples, found to be exclusive in iCCA and mutually exclusive with the canonical *KRAS/BRAF* mutations. This

stresses the urgent need for evaluation of the efficacy of FGFR inhibitors such as ponatinib, pazopanib or lenvantinib. Although less frequent, alterations in several FGFRs as well as FGF19 are also observed in HCC [89]. Despite the failure of brivanib to improve overall survival of HCC patients [90], several other agents with effect on the FGFRs are currently under clinical evaluation [73]. Members of the canonical RAS/MAPK signaling cascade downstream from FGFRs, RTKs such as MET and ERBBs, as well as growth factor receptors such as IGFRs and PGDFRs, are attractive drugable targets and often deregulated in iCCA (reviewed in [49]). Currently, MEK1 is under intensive investigation in both CCA and HCC, representing a promising intermediate in the RAS/RAF/MEK/ERK pathway that bypasses issues of drug response related to mutations in *KRAS* and *BRAF,* and therefore does not restrict the treatment to patients with wild-type *RAS* gene status, as is the case for anti-EGFR therapies. This is particular important for patients diagnosed with CCA since mutations in *KRAS*, for example, are relatively prevalent (up to 20% in iCCA with an increasing incidence in pCCA and towards the pancreas). Several clinical trials evaluating drugs targeting MEK (selumetinib [AZD6244] [91], ARRAY-438162 and GSK1120212) are either ongoing or concluded.

The most prevalent activating mutation detected in iCCA is found in the gene PTPN3 with recurrent somatic mutations observed in several members of the PTP family of protein tyrosine phosphatases. Mutations in the PTP family have been found in more than 50% of iCCA cases [60]. PTPN3 or the members of the PTP family were also suggested as likely candidates responsible for the deregulated receptor tyrosine kinase expression and signaling often observed in CCAs from patients with poor outcome. However, mutations in *PTPN3* and its increased expression were found to be prognostic significant as a marker for early detection in tumors with no lymph node or intrahepatic metastases, and correlated with risk of post-operative tumor recurrence.

Unresolved issues

The dawn of NGS technologies and the accompanying opportunities have not only generated great opportunity, but also considerable challenges for translational applications in cancer research. Besides the associated costs, the amount of generated data and depths of analyses currently exceed our ability to interpret and subsequently transform the data into useful clinical information [92]. Application of the technologies for translational applications requires both training and interaction of bioinformaticians and healthcare providers, as well as patients whose backgrounds on the genetic basis of a disease are highly variable. The difficulty in establishing ethically and clinically valid standards for the appropriate use of the technology and integration in our diagnostic pipeline remains another major burden [93]. In liver cancer, several other specific problems have to be overcome for successful implementation of NGS technologies into clinical routine.

First, the application of translational genomics requires availability of well-preserved specimens in addition to routine pathology, which highlights the need for mandatory biopsies and, consequently, adaptation of current guidelines [94]. Further, molecular and cellular (e.g., stromal infiltration) tumor heterogeneity as well as resistance to current treatment modalities might require sequential analysis of the respective genomic landscape to successfully guide individual treatment decisions. The underlying liver disease as well as the cellular diversity might require additional sampling of the noncancerous microenvironment, which further complicates decisions. Further, the spectrum of molecular alterations is highly dependent on the ethnicity, etiology and regional background of the liver disease. As a consequence, a significant molecular heterogeneity is increasingly recognized in the current studies, confirming that a clear oncogene addiction cannot be assumed for liver cancer. This is a concern for future clinical trial designs. Consequently, large cohorts are required to obtain a sufficient amount of samples for identification and treatment of patients that share a similar molecular profile. Given the failure of recent Phase III trials, funding might, therefore, be particularly problematic for NGS technologies in liver cancer. Beyond any doubt, to achieve the paradigm shift towards precision medicine in liver cancer, multi-center consortia and international collaborations are crucial for success.

Conclusion & future perspective

In primary liver cancer the underlying genetic diversity is fostered by chronic inflammation of a permissive tumor microenvironment.

Technical progress in the past few years has greatly advanced our understanding of tumor biology, increasing our knowledge of the molecular complexity and intratumoral heterogeneity. In liver cancer, it remains to be seen if NGS, which holds great promise for individualized medicine, will be implemented in future patient management. Integration of multiple molecular layers unique to the individual will ultimately embrace a more meaningful impact for clinical applications. However, to achieve this challenging goal and to fully utilize the potential of NGS for the understanding of the liver cancer genome, systematic application of genome-wide analyses into clinical trials will be necessary. The pursuit of genomic alterations in human diseases has enabled the discovery of novel driver genes, mutations and oncogenic-addition networks, which, for some solid tumors such as breast, colon, lung and melanoma, has resulted in advancement in clinical-decision and patient outcome. This clinical development has not reached liver cancer, where molecular classification has not been extended to patient stratification in the clinical setting. A shift in this paradigm, enriching patients on the background of a molecular profile, signals a change in the recent Phase III trial, evaluating tivantinib (against MET oncogene) in patients with tumors overexpressing MET [95]. In this context, high-throughput analyses should be adapted for diagnostic and prognostic classification, dissecting the mechanism of acquired resistance and predicting recurrence to ultimately contribute to treatment decisions and new drug development [96]. If this endeavor could be achieved it is highly possible that NGS technology will continue to transform cancer research, leading to a comprehensive understanding of individual tumor genetics. In this review, the authors have outlined the recent NGS discoveries that demonstrate many genetic variants unique to the specific tumor type, such as *CTNNB1* in HCC and *KRAS*, *IDH1* or *FGFR2* fusion genes in iCCA. Importantly, common alterations such as *TP53*, and various novel drugable genetic aberrations in, for example, chromatin-remodeling factors, have been highlighted. Furthermore, collection of the diverse information in large databases to connect genomic findings with clinical parameters will be of central importance. The near future will show if liver cancer clinically is braced for NGS.

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