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Hepatocyte ploidy and pathological mutations in hepatocellular carcinoma: impact on oncogenesis and therapeutics

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Abstract: Hepatocellular carcinoma (HCC) occurs in the chronic liver inflammation such as viral hepatitis, alcoholic and non-alcoholic steatohepatitis. While anti-viral treatment has been significantly improved, the prevalence of HCC remains high and treatment is still challenging. The continuation of hepatocyte death, inflammation, and fibrosis leads to the accumulation of gene alterations, which may trigger carcinogenesis. Hepatocytes are a unique cell type having more than one complete set of 23 chromosomes, termed polyploidy. Due to gene redundancy, hepatocytes may tolerate lethal mutations. Next generation sequencing technology has revealed gene alterations in HCC related to telomere maintenance, Wnt/β-catenin pathway, p53 cell-cycle pathway, epigenetic modifiers, oxidative stress pathway, PI3K/AKT/mTOR, and RAS/RAF/MAPK pathway with or without a chromosomal instability. Some type of driver gene mutations accumulates in hepatocytes and breaks the orchestration of excessive copies of chromosomes, which may lead to unfavorable gene expressions and fuel tumorigenesis. Recently, molecular targeted drugs, developed with the aim of interfering with these signaling pathways, are being used for HCC patients in the clinics. Therefore, a deeper understanding of hepatocyte ploidy and genetic or epigenetic alterations is indispensable for the establishment of novel therapeutic strategies against HCC.

***Keywords*:** hepatocellular carcinoma (HCC), ploidy, mutation, next generation sequence, molecular targeted drug

Introduction

Hepatocellular carcinoma (HCC) is a primary liver tumor which is the fifth leading cause of cancer-related death in Japan. Most HCC is found in patients with liver cirrhosis or chronic liver injury, such as viral infection [hepatitis B virus (HBV), hepatitis C virus (HCV)], alcoholic injury, non-alcoholic fatty liver disease (NAFLD) and autoimmune diseases including primary biliary cholangitis and autoimmune hepatitis. WHO estimated that 53% of HCC occurrence is found in patients with HBV infection, and another 25% in patients with HCV infection. On the contrary, in Japan, approximately 65% of HCC cases were caused by HCV infection and 15% by HBV infection (*1*). The recent multi-institutional nationwide survey in Japan reported that the proportion of non-viral liver cirrhosis caused by alcohol intake or non-alcoholic steatohepatitis (NASH) has increased (*2*). To prevent from chronic injury in liver according to its local circumstances, measures against viral infections control of viral infection, and lifestyle modification including reduction of alcohol consumption, healthy diet and physical exercises are feasible for secondary prevention. Although the direct acting antiviral (DAA) therapy for HCV and the nucleotide analogs for HBV have been widely used, HCC is still one of the few neoplasms showing the greatest increase in mortality in the United States during the past two decades (*3*). Moreover, the recent systematic review, utilizing economic studies published for a decade from 2008, demonstrated that HCC incidence is approximately 100 times higher among patients with chronic hepatitis/cirrhosis and one third of them also diagnosed with advanced disease (*4*).

Chronic injury and inflammation stimulate proliferation of cells and accumulate gene mutations resulting in carcinogenesis, which occurs not only in hepatitis, but also in several inflammatory diseases including pancreatitis, colitis, esophagitis, cholangitis, and gastritis. Many researchers have aimed to elucidate the mutations that drive oncogenesis in the liver for decades. The recent development of next-generation sequencing (NGS) technology provides us with a better understanding of the mutational landscape during liver oncogenesis and the correlation with pathological appearances and clinical prognosis (*5,6*).

The fourth edition of guidelines for HCC treatment in Japan was recently published (*7*). The recommended treatments are determined by liver functional reserve, extrahepatic metastasis, vascular invasion, tumor number, and tumor size. Radiofrequency or microwave ablation, liver resection, or liver transplantation, all

potential curative therapies for HCC, should be the first-line treatments when the tumors are limited to the liver within an early stage criterion. For patients who are not candidates for these above treatments, locoregional treatments, including transarterial chemoembolization (TACE), transarterial radioembolization (TARE), and stereotactic body radiation (SBRT) are recommended. In the guideline, molecular targeted therapy is a newly added recommendation for HCC patients qualified as Child-Pugh A liver functional reserve with extrahepatic metastasis. Sorafenib is the first approved molecular targeted drug for advanced-stage or unresectable HCC which inhibits tyrosine kinase of VEGF receptors, PDGFR, and Raf kinases. The recent guidelines released by the American Association for the Study of Liver Diseases (AASLD), the European Association for the Study of the Liver and European Organization for Research and Treatment of Cancer (EASL-EORTC) , and the Japan Society of Hepatology (JSH) recommend sorafenib as a systemic therapy (*8,9*). Therapeutic developments are also being made in the field of systemic chemotherapy. Several clinical trials using other molecular targeted drugs including tyrosine kinase inhibitor and immune checkpoint inhibitor have been undertaken for HCC treatment. The molecular understanding of HCC may guide in developments of promising cancer therapy for patients with advanced stage HCC. Thus, a molecular understanding of HCC has become more important in clinical practice .

In this review, we briefly outline the recent research on HCC from three perspectives: *i*) biology of hepatocyte heterogeneity (especially ploidy) with adaptive and protective effects for injury and oncogenesis, *ii*) pathological mutations to allow HCC oncogenesis, and *iii*) its therapeutic implications.

Hepatocyte polyploidy and carcinogenesis

A characteristic appearance of hepatocytes is polyploidy, which is an increase in the number of chromosome sets per cell. A population of hepatocytes has two nuclei in one cell with a difference in DNA amount per nucleus. For instance, a tetraploid hepatocyte could have a mono-nucleated tetraploid (4N) nucleus or two bi-nucleated (2N+2N) diploid nuclei (Figure 1). The accumulation of chromosomes happens drastically around weaning and remains during ageing, mainly because of cytokinesis failure. Liver injury, such as surgical resection, toxic stimulation, metabolic iron and copper overload, telomere attrition, chronic viral infection with HBV and HCV, or oxidative stress has been reported to induce polyploidization (*10,11*). Additionally, many age-related diseases including arterial hypertension, hyperthyroidism, metabolic disorders and cancer have an association with polyploid accumulation (*11*). These findings have raised the question of whether the polyploidy of hepatocytes is beneficial or detrimental

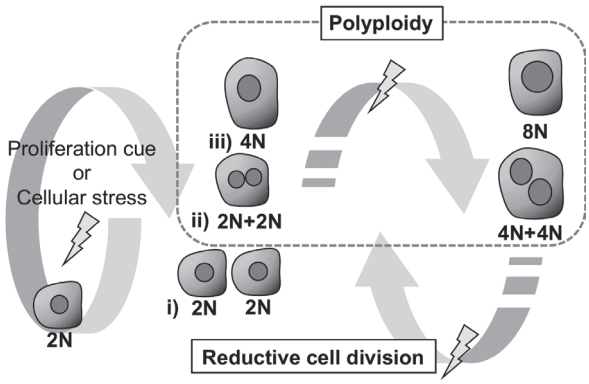

Figure 1. The polyploidization of hepatocyte and normal cell division. Under proliferative demands or cellular stresses, the hepatocyte undergoes duplication of its DNA and generates three different types of ploidy states: *i*) The conventional cell division generates two daughter cells with same DNA content of parent cell, *ii*) Cytokinesis failure results in bi-nucleated hepatocyte with same DNA content in each nucleus, and *iii*) Endoreplication generates mono-nucleated hepatocyte harboring double. Another cue of cell cycle drives accumulation of ploidy or even reductive cell division without DNA synthesis. N means number of haplotypes.

for homeostasis. Aneuploidy especially, including multiplication of complete sets of chromosomes and excessive or deficit parts of chromosomes (*e.g*. trisomy or monosomy), has been frequently identified in cancer cells and is regarded as a risk factor for chromosomal instability. The functions of polyploidy in the liver either as physiological conditions or pathological responses are still largely unknown.

Studies of plants demonstrate that polyploidy contributes to species diversity, which provides an evolutionary advantage in response to its environment (*12*). Mammalian polyploidy is rare and is limited to cardiomyocytes of the heart, trophoblasts of the placenta, megakaryocytes in bone marrow, acinar cells of the pancreas, and hepatocytes of liver under physiological circumstances. The idea that these polyploidy states contribute to cellular diversity which provides a selective advantage in response to injuries is plausible. Aneuploidy itself is not sufficient to generate neoplastic chromosomal instability (CIN), but modulates cellular metabolic and transcriptional programs, such as higher glucose and/or glutamine consumption, changes in proteins involved in the cell cycle, ribosome biogenesis, endoplasmic reticulum, Golgi apparatus, lysosomes, membrane metabolism, the major histocompatibility complex (MHC) proteins, and antigen processing (*13*). Polyploid cells like cardiomyocytes, megakaryocytes and trophoblast giant cells, have been accepted as terminal differentiated and functionally mature and considered less stem cell-like. Interestingly, Duncan *et al*. demonstrated that polyploid hepatocytes could contribute to regeneration through a "ploidy conveyor", in which proliferating polyploid hepatocytes generate a highly diverse population of daughter cells with multiple numerical chromosome

imbalances as well as uniparental origins (*14*). In the other study utilizing fumarylactate hydrolase (Fah) deficient mice, in which the liver is chronically injured by the accumulation of metabolite, hepatocytes with a heterozygous mutation of the homegentisic acid dioxygenase (Hgd) gene, upstream of Fah, show loss of the chromosome with the Hgd gene and escape from toxic metabolite synthesis (*13,15*). This phenomenon clearly demonstrates that the selection of specific aneuploid karyotypes can result in the adaptation of hepatocytes to chronic liver injury.

The impact of polyploidy on tumorigenesis has also been reported. Aneuploidy is mostly caused by deregulation of the spindle assembly checkpoint (SAC). Some mouse models with dysfunction of SAC genes show resistance to tumorigenesis (*13*). These findings support the beneficial role of excessive chromosomes. On the contrary, the study utilizing TP53 null mice, of which tetraploid mammary epithelial cells have more potent malignant tumor formation than diploid, suggested that polyploidy is a gateway to tumorigenesis (*16*). In terms of heterozygosity, multiple copies of a tumor suppressor gene allele would be protective against loss of heterozygosity (LOH) which leads to oncogenesis. Indeed, Zhu and his colleagues demonstrated that a transcriptional factor responsible for cell cycle, E2f8 knockdown in the liver resulted in diploid hepatocytes which became vulnerable to DNA damage by diethylnitrosamine (DEN), and developed liver tumors; while an F-actin binding protein responsible especially for cytokinesis, Anillin knockdown in the liver resulted in higher polyploidy hepatocytes which did not develop tumors (*17,18*).

A clinical study shows a reduced proportion of binucleated hepatocytes in non-tumor liver tissue adjacent to the HCC, and suggests that non-tumoral hepatocytes have a susceptible condition to LOH (*19*). Interestingly, a TP53 mutation has been seen in hyperploid hepatocytes or multinucleated hepatocytes in different studies (*5,19*). Cytokinesis failure and tetraploidization can activate the Hippo tumor suppressor pathway *via* extra centrosomes, which resulted in p53 stabilization and inhibition of cell growth when p53 was intact (*20*). Taken together, polyploid status appears to be protective against oncogenesis until TP53 is disrupted, in which case it becomes promotive toward oncogenesis thereafter. These evidence remind us that the combination of mutations would result in different outcomes. Therefore, a comprehensive understanding for HCC oncogenesis based on the landscape of gene mutations is crucial for treatment.

Genetic alterations in liver cirrhosis and HCC

Many liver cancers exhibit high degrees of genomic instability, which is roughly categorized as mitotic error-mediated chromosome instability (CIN) and DNA metabolism defect-mediated microsatellite instability (MIN). While MIN may have a minor role in hepatocarcinogenesis, CIN is one of the most frequent abnormalities in HCC (*21*). More than half of HCC (58-86%) have been harboring a copy number gain at 1q where five cancer genes, BCL9, ARNT, TPM3, MUC1 and NTRK1, and cell-cycle related genes, CHD1L, CKS1B, JTB and SHC1 located (*22,23*). Chromosome 8q is the second and is seen in half of HCC, which results in amplification of MYC, DDEF1 and MLZE (*24*). These amplifications as gain-of-functions are associated with patients' prognosis.

Micronuclei (MNi) are extra-nuclear bodies that contain damaged chromosome fragments isolated from the parent nucleus after cell division. MNi are considered sensitive markers of genotoxic damage and chromosomal instability in cirrhosis and HCC (*25*). A large number of rearrangements in a restricted region of chromosome known as chromothripsis are processed in these MNi. Therefore, a part of chromosomal instability is a consequence of MNi formation. Recent studies also highlight the high frequency of the chromothripsis throughout malignant tumors including HCC (*26,27*).

The recent intensive studies of whole-exome and whole-genome sequencing have identified mutations responsible for HCC oncogenesis and its pathological character. In these studies, the commonly observed gene mutations are related to telomere maintenance, Wnt/β-catenin pathway, p53 cell-cycle pathway, epigenetic modifiers, oxidative stress pathway, PI3K/AKT/mTOR, and RAS/RAF/MAPK pathway (Figure 2).

TERT promoter

Chronic liver injury leads to shortened telomere length and results in cirrhosis. Telomerase reverse transcriptase (TERT) is an enzyme that elongates telomeres with telomerase RNA component. The reactivation of telomerase is seen in approximately 90% of HCC. TERT promoter mutations have been found in 12-48% of HCCs and more frequently in HCCs from HCV infection and alcohol intake than HBV infection (*28*). However, integration of HBV DNA into the TERT promoter region contributes to overexpression of TERT, resulting in cell immortalization in 15-20% of HBV-associated HCCs (*29-31*). The TERT promoter mutations were also found in 6% of low-grade dysplastic nodules and 19% of high-grade dysplastic nodules in patients with cirrhosis (*32*) and appear to be required for hepatocellular adenoma to carcinoma development in non-cirrhosis patients (*33*). Thus, mutation in the TERT promoter is one of the important early events of HCC oncogenesis as a tumor initiation, and has close interactions with the MYC, Wnt/β-catenin pathway, and NF-κB pathway (*6,34,35*).

Wnt/β-catenin pathway

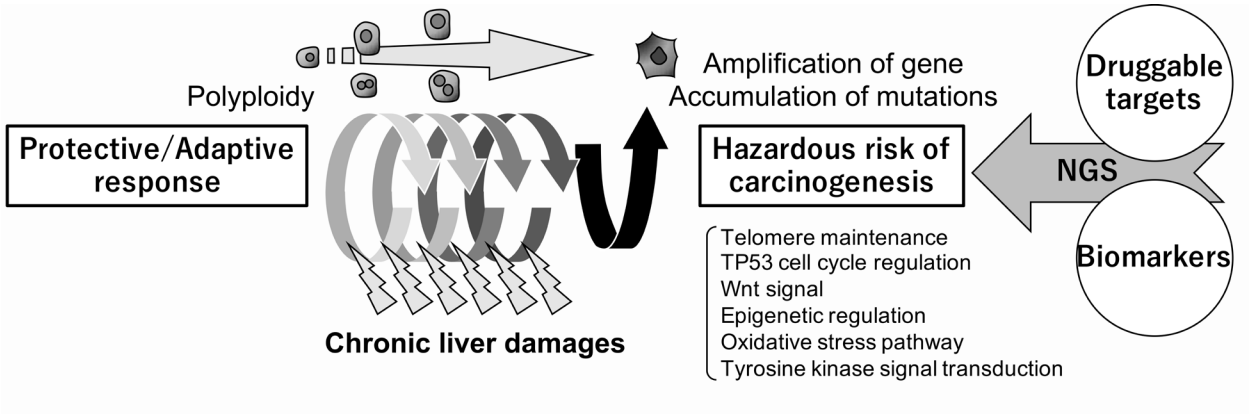

Figure 2. The gene alterations of hepatocytes under chronic liver injury and hazardous mutations of carcinogenesis. The polyploidy of hepatocytes plays a protective role against chronic liver injury. The multiple dysregulation of important genes by accumulation of gene alterations, including mutations and amplifications, could contribute to carcinogenesis. Next-generation sequencing (NGS) has revealed some key mutations in hepatocellular carcinomas. These findings would be helpful for the identification of "druggable" targets and biomarkers.

Wnt signaling is responsible for cell motility, dedifferentiation, and proliferation (*36*). The activating mutation of β-catenin (CTNNB1) and inactivating mutation of AXIN1, one member of β-catenin destruction complex, have been found in 11-37% and 5-15% of HCCs, respectively (*28*). While CTNNB1 and TP53 mutations have been found to be mutually exclusive, the CTNNB1 mutation coincided with TERT promoter mutations in early oncogenesis of HCC that is characterized as a CTNNB1 tumor subgroup, which is usually large and well-differentiated with less inflammatory infiltrates (*5*). The correlation between Wnt/β-catenin pathway and absence of T cell infiltration has been reported in melanoma and recently in HCC (*37,38*).

TP53 cell-cycle pathway

The dysfunction of TP53 resulting from mutations and/or repression by HBx has been detected in approximately 12-48% of HCCs, and with a higher frequency in advanced tumors (*28*). These mutations are characterized as a TP53 tumor HCC subgroup, which is likely to be poorly differentiated, associated with vascular invasion, multinucleated, and pleomorphic (*5*).

Epigenetic modifiers

Mutations in the chromatin remodeling enzyme ARID1A and ARID2 are found in 4-17% and 3-18% of HCCs, respectively (*28*). These mutations are closely related with transcription factor E2F and cyclin-dependent kinase inhibitor p21.

Mutations in the histone methylation MLL, MLL2, MLL3, and MLL4 have also been found. Inactivation of chromatin remodeling enzymes have been detected mostly in HCCs with liver disease from alcoholism (*39*).

Oxidative stress pathway

The mutations repeatedly identified in the oxidative stress pathway, such as NFE2L2 and KEAP1 lead to prolonged cell life and tumor growth (*39*).

PI3K/AKT/mTOR and RAS/RAF/MAPK pathway

Some HCCs show mutations in tyrosine kinase receptor pathways including PI3K/AKT/mTOR and RAS/RAF/MAPK. EGFR, VEGFR and PDGFR, lying upstream of these pathways, are targets of "molecular targeted drugs" such as sorafenib. High level amplification of VEGF signaling has been identified in 7-10% of HCCs (*40,41*). Contrary to rare mutations in KRAS, RAS/RAF/MAPK signaling is activated in half of early and almost all advanced HCC, as a result of Epidermal Growth Factor (EGF), Insulin growth factor (IGF) and MET activation (*42,43*). PI3K/AKT/mTOR pathway is activated in half of HCCs (*44*). IGFR signaling, upstream of PI3K, is activated in 20% of HCC through IGF2R allelic loss, ligand overexpression, or dysfunction of IGF binding proteins (IGFBPs) (*45*).

Others

IL-6/JAK/STAT and TGF-β are seen in 9% and 5% of HCC. Other mutations or copy number variations have been found in FGF19, VEGFA, MYC, CCND1, IGF, Hedgehog, and MET pathways (*46*). PTEN and CDKN2A (P16INK4A) are frequently deleted.

Many other gene alterations are involved in HCC oncogenesis and are closely associated with each other. To simplify these complicated interactions, researchers have classified HCCs based on their mutation signatures, which are aligned with HCC-related risk factors, such as age, sex, race, HBV infection, tobacco and alcohol consumption, and sporadic mutations by aflatoxin B1 in fungal contaminated food or aristolochic acid derived from Chinese herb (*47*). In clinical practice, the 5-gene score, based on combined expression level

of HN1, RAN, RAMP3, KRT19, and TAF6 is proposed to predict the disease-specific survival after resection (*48*). Moreover, for cohorts with different etiology, other studies have proposed prognostic signatures in transcripts of different gene sets, microRNA or DNA methylation (*49-53*). Cholangiocarcinoma, another type of primary tumor that develops in the liver, has also been studied in whole genome sequences, revealing that different gene mutation including KRAS, BRAF, BAP1, SMAD4, IDH1, and IDH2 are involved (*54-56*).

Another gene signature study has also highlighted the heterogeneity of even hepatic cancer stem cell (CSCs) marker EpCAM, CD133, CD24 and triple positive cells with single cell level which can be used for HCC patient survival prediction (*57*). Further analyses with single cell resolution could help establish a model of cancer evolution and identify targets for therapy.

The study on genomic mutations of Mongolian HCC, which reported new driver genes such as GTF2IRD2B, PNRC2 and SPTA1 that are closely associated with hepatitis D viral infections (*58*), is a reminder of the importance of stratification by precise etiological characteristics of cohorts for understanding the interpatient heterogeneity of HCC.

Although studies on gene signatures of non-tumor livers have yielded information on prediction of HCC early recurrence, the question of how and which mutations are accumulated, and in which chronological order before aggressive tumor growth has yet to be fully answered. Recently, a comparative study between the outside and inside of nodule-in-nodule tumors, along with regenerative nodules and non-tumor areas was undertaken. The study showed that mutations, CNV, and epigenetic modification in some of the previously reported HCC-related driver genes, including TERT and TP53, have been found during early stage HCC (*59*). As series of gene alteration accumulated in the liver are shown to closely associate with the phenotype of HCC. Further investigations on both unique chromosomal regulation in hepatocyte and heterogenous accumulation of genetic alterations in HCCs with different etiology are indispensable for better understanding of HCC.

Molecular targeted drugs for HCC: Sorafenib and beyond

Because underlying mutations play important roles in oncogenesis of the liver, inducing deleterious functions in them has become an attractive strategy for cancer therapy. Molecular targeted therapies differ from standard chemotherapy, which are characterized by targeting specific enzymes, growth factor receptors and signal transducers, thereby interfering with a variety of oncogenic cellular processes without adverse effect on normal cells (*60*).

A tyrosine kinase inhibitor, sorafenib, has been confirmed to improve median overall survival in two multicenter RCTs: the Sorafenib Hepatocellular Carcinoma Assessment Randomized Protocol (SHARP) trial, including mostly Caucasians with HCV infection, and the Asia Pacific trial including Asians with HBV infection (*61,62*). The proposed targets of sorafenib broadly covers the RAF/MAPK/ERK pathway, VEGFR, PDGFR, and anti-apoptotic protein Mcl-1 (*63*). Unfortunately, molecular targeted therapies, including sorafenib, still provide insufficient outcome in terms of the overall survival elongation. To overcome this, therapy for HCC should include personalized modification and selection of drugs (*63*). The SHARP trial shows a non-significant trend towards higher survival benefit of sorafenib treatment with high c-kit or low plasma HGF levels. Therefore, the predicting the outcome of sorafenib treatment is not so simple. It requires an identification of poor/outstanding responding patients by using biomarkers and characterization of the HCC with pathological alterations including gene mutation.

The phase 3 STORM trial and BIOSTORM study on the efficacy of sorafenib as an adjuvant therapy following surgical resection or local ablation, revealed a gene signature of 87 poor prognosis genes and 59 good prognosis genes (*64*). In enrichment analysis, sorafenib recurrence free survival (RFS) responders showed downregulation of pathways indicative of poor prognosis such as KRAS, activation of EIF2 signaling, oxidative stress responses, immune-related processes, and upregulation of bile acid and lipid metabolism-related pathways. Of note, all these molecular traits were also present in the non-tumor adjacent tissue. Although VEGF-A gene copy number is suggested as a response predictor by retrospective observation study (*65*), VEGF-A focal amplification correlates with tumor satellites and microvascular invasion but not with recurrence prevention in the study.

The molecular targeted drugs already approved for clinical use interfere with the PI3K/AKT/mTOR and RAS/RAF/MAPK pathways, which are also involved in liver regeneration. Such drugs may lead to unfavorable effects on non-tumor regions of parenchyma damaged by chronic liver injuries. Of importance is not only the identification of suitable cohorts for treatment with the existing drugs, but also the development of new drugs that interact with malignant cells specifically. The proteomic profiling of 110 paired tumor and non-tumor tissues derived from the patients with HBV-related early HCC have identified sterol *O*-acyltransferase 1 (SOAT1) as a potential target (*66*). Using different cohorts, this approach may reveal other candidates as "druggable" targets leading to development of specific therapies for certain cohorts and/or robust therapies for all HCC patients.

Several negative trials have been reported for a decade since sorafenib approval. Regorafenib, an oral multi-kinase inhibitor, has shown significance in overall survival, compared with placebo in a phase

III (RESORCE) study, as a second-line therapy after sorafenib treatment (*67*). Recently in succession, lenvatinib in the REFLECT study, ramucirumab in the REACH-2 study, and cabozantinib in the CELESTIAL study have demonstrated their efficacy for HCC. In Japan, lenvatinib as well as sorafenib are recommended as first-line therapies for unresectable advanced HCCs. Regorafenib is recommended as second-line therapy, after sorafenib, for patients with HCC showing disease progression (*7*). Lenvatinib is an oral multi-kinase inhibitor that targets VEGF receptors, FGF receptors, PDGF receptor α, RET and KIT, and is non-inferior to sorafenib in overall survival as a first-line therapy (*68*). Ramucirumab is a human IgG1 monoclonal antibody that blocks signal transduction of VEGFR2, and shows improved overall survival in sorafenib-treated patients with α-fetoprotein concentrations of 400 ng/mL or greater (*69*). Cabozantinib targets tyrosine kinases including EGFs, MET, and AXL, and shows significantly longer overall survival than placebo as a second line treatment following sorafenib (*70*). As with sorafenib, the other molecular targeted drugs have been subjected to subgroup analyses and molecular assays including genetic sequences, which should provide cues to improve HCC treatment.

Genes involved in HCC chemo-resistance

Drug resistance results from the reduction of drug intake, enhancement of drug efflux and metabolic degradation, as well as mutations in drug targets. Based on the comparison of blood samples from 3 different responder types, such as extreme, strong and poor, six non-synonymous SNVs were found in four ADME (Absorption, Distribution, Metabolism, and Excretion) related genes: ABCB1, FMO3, and SLC15A2 (*71*). These molecules are important in terms of drug resistance to antibiotic and anticancer therapy. ABCB1 codes for one of the super families of ATP-binding cassette transporters. FMO3 codes for a flavin-containing monooxygenase which is a member of an important class of drug-metabolizing enzymes. SLC15A2 codes for a member of a family of proton-coupled peptide transporters. Among these genes, the single nucleotide polymorphism of ABCB1 was also related to sorafenib sensitivity in HCC patients (*72*). The ATP-binding cassette (ABC) transporter superfamily is one of the largest classes of transporters, which translocates many substrates including nutrients, viruses, and waste products through membranes of cells. Members of the ABC transporter family are present in organisms from all kingdoms of life, and play essential roles in maintaining homeostasis. Recent studies also repeatedly identified another member of the ABC transporter family, ABCC1 (MRP2); of which SNPs show altered transport activity for sorafenib, and efflux of paclitaxel and doxorubicin (*73,74*). The solute carrier (SLC) transporter superfamily, one of the counter parts of ABC transporters, imports solutes from the extracellular milieu into the cell depending on concentration gradient. The SLC superfamily, genetically heterogeneous with more than 200 exonic SNVs, is associated with clinical drug response or toxicity (*75*). Another member of SLC transporters, SLC22A1 (OCT1) is also associated with response to antitumor therapy with sorafenib (reviewed by Cabral *et al*. (*76*)). Intracellular drug metabolism Phase I and II enzymes including FMO3, cytochrome P450 (CYPs), and UGT (uridine diphosphate glucuronosyltransferase) play an important role in homeostatic control of lipophilic endobiotics, detoxification of xenobiotics and drug transportation (Phase III). Sorafenib is metabolized by CYP3A4 and UGT1A9, in which polymorphism leads to poor metabolism and is associated with sorafenib-induced severe toxicity (*76*). Awareness of genetic polymorphisms in metabolizing enzymes therefore, is also indispensable for precision medicine.

Future perspectives: towards the stage of immuno-therapy

In the IMbrave 150 phase III trial, combination therapy utilizing two monoclonal antibodies, atezolizumab and bevacizumab, against PD-L1 and VEGF-A respectively, showed a significantly improved survival rate of unresectable HCC compared to sorafenib therapy (*77*). To date, clinically available molecular targeted drugs are limited. Combination therapy including immunotherapy such as blockade of immune checkpoint CTLA-4 and PD-1, neoantigen, and CAR (chimeric antigen receptor) T cell therapy would be feasible alternatives.

Aneuploidy, the harboring of an abnormal number of chromosomes, in several tumors has been reported as a predictive biomarker for efficacy of immune checkpoint inhibitor (*78*). A recently conducted study on the relationship between copy number alterations (CNAs) and immune profiles of HCC, found that higher levels of broad CNAs resulting from aneuploidy show less immune-cell infiltration and are regarded as the primary reason for resistance to immune therapy (*79*). This CNAs and immune-phenotype relationship was seen in dysplastic nodules and early HCC. The enriched copy number loss, including HLA-DQB1, found in high broad CNA tumors appears to be one reason. The study also shows that 44-68% of HCC display polyploid status, which enriches high levels of broad CNAs. These chromosomal abnormalities, along with Wnt/β-catenin and JAK/STAT pathway signatures discoveries, should be prioritized in the development of future immunotherapies for HCC.

Neoantigens expressed specifically in tumors, including proteins derived from viruses or mutated genes, are most attractive targets for cancer immunotherapy. To date, clinical trials targeting AFP,

GPC3, TERT or MRP3 have been conducted as phase I or II (reviewed by Lu *et al*. (*80*)). These limited studies could identify new candidates.

To translate this rapidly growing knowledge for clinical practice, blood samples from patients with HCC have been subjected to genetic analysis. Droplet digital PCR (ddPCR) is highly sensitive and enables detection of small amounts of DNA. Liquid biopsy specimens contain genetic information in circulating tumor cells (CTCs) or cell free nucleic acids (cfNAs) including DNA, mRNA, miRNA and lncRNA. Total cfDNA levels themselves appear to be a biomarker for HCC screening, monitoring of treatment, and prediction of recurrence (reviewed by Bubu *et al*. (*81*)). Establishment of a system for sample collection, isolation and preservation, especially for liver and tumor tissues, is still challenging.

Herein, we review the biology of hepatocyte ploidy, pathological mutations in HCC oncogenesis, and their therapeutic implications. The breakthrough of next generation sequencing has provided a huge amount of information in understanding genetic alteration of cancer. However, such information is fragmented and needs to be pieced together for discovery of missing links. Molecular targeted drugs and immune checkpoint inhibitors may revolutionize the practice guidelines for HCC therapies in the near future.

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References

1. Tanaka J, Akita T, Ko K, Miura Y, Satake M; Epidemiological Research Group on Viral Hepatitis and its Long -term Course, Ministry of Health, Labour and Welfare of Japan. Countermeasures against viral hepatitis B and C in Japan: An epidemiological point of view. Hepatol Res. 2019; 49:990-1002.
2. Enomoto H, Ueno Y, Hiasa Y, *et al*. Transition in the etiology of liver cirrhosis in Japan: a nationwide survey. J Gastroenterol. 2020; 55:353-362.
3. Sia D, Villanueva A, Friedman SL, Llovet JM. Liver cancer cell of origin, molecular class, and effects on patient prognosis. Gastroenterology. 2017; 152:745-761.
4. Aly A, Ronnebaum S, Patel D, Doleh Y, Benavente F. Epidemiologic, humanistic and economic burden of hepatocellular carcinoma in the USA: a systematic literature review. Hepat Oncol. 2020; 7:HEP27.
5. Calderaro J, Couchy G, Imbeaud S, Amaddeo G, Letouzé E, Blanc JF, Laurent C, Hajji Y, Azoulay D, Bioulac-Sage P, Nault JC, Zucman-Rossi J. Histological subtypes of hepatocellular carcinoma are related to gene mutations and molecular tumour classification. J Hepatol. 2017; 67:727-738.
6. Dhanasekaran R, Nault JC, Roberts LR, Zucman-Rossi J. Genomic medicine and implications for hepatocellular carcinoma prevention and therapy. Gastroenterology. 2019; 156:492-509.
7. Kokudo N, Takemura N, Hasegawa K, *et al*. Clinical practice guidelines for hepatocellular carcinoma: The Japan Society of Hepatology 2017 (4th JSH-HCC guidelines) 2019 update. Hepatol Res. 2019; 49:1109-1113.
8. European Association For The Study Of The Liver; European Organisation For Research And Treatment Of Cancer. EASL-EORTC clinical practice guidelines: management of hepatocellular carcinoma. J Hepatol. 2012; 56:908-943.
9. Heimbach JK, Kulik LM, Finn RS, Sirlin CB, Abecassis MM, Roberts LR, Zhu AX, Murad MH, Marrero JA. AASLD guidelines for the treatment of hepatocellular carcinoma. Hepatology. 2018; 67:358-380.
10. Wang MJ, Chen F, Lau JTY, Hu YP. Hepatocyte polyploidization and its association with pathophysiological processes. Cell Death Dis. 2017; 8:e2805.
11. Donne R, Saroul-Aïnama M, Cordier P, Celton-Morizur S, Desdouets C. Polyploidy in liver development, homeostasis and disease. Nat Rev Gastroenterol Hepatol. 2020; 17:391-405.
12. Nieto Feliner G, Casacuberta J, Wendel JF. Genomics of Evolutionary Novelty in Hybrids and Polyploids. Front Genet. 2020; 11:792.
13. Simonetti G, Bruno S, Padella A, Tenti E, Martinelli G. Aneuploidy: Cancer strength or vulnerability? Int J Cancer. 2019; 144:8-25.
14. Duncan AW, Taylor MH, Hickey RD, Hanlon Newell AE, Lenzi ML, Olson SB, Finegold MJ, Grompe M. The ploidy conveyor of mature hepatocytes as a source of genetic variation. Nature. 2010; 467:707-710.
15. Duncan AW, Hanlon Newell AE, Bi W, Finegold MJ, Olson SB, Beaudet AL, Grompe M. Aneuploidy as a mechanism for stress-induced liver adaptation. J Clin Invest. 2012; 122:3307-3315.
16. Fujiwara T, Bandi M, Nitta M, Ivanova EV, Bronson RT, Pellman D. Cytokinesis failure generating tetraploids promotes tumorigenesis in p53-null cells. Nature. 2005; 437:1043-1047.
17. Zhang S, Zhou K, Luo X, Li L, Tu HC, Sehgal A, Nguyen LH, Zhang Y, Gopal P, Tarlow BD, Siegwart DJ, Zhu H. The Polyploid State Plays a Tumor-Suppressive Role in the Liver. Dev Cell. 2018; 44:447-459.e5.
18. Lin YH, Zhang S, Zhu M, *et al*. Mice With Increased Numbers of Polyploid Hepatocytes Maintain Regenerative Capacity But Develop Fewer Hepatocellular Carcinomas Following Chronic Liver Injury. Gastroenterology. 2020; 158:1698-1712.e14.
19. Bou-Nader M, Caruso S, Donne R, *et al*. Polyploidy spectrum: a new marker in HCC classification. Gut. 2020; 69:355-364.
20. Ganem NJ, Cornils H, Chiu SY, O'Rourke KP, Arnaud J, Yimlamai D, Théry M, Camargo FD, Pellman D. Cytokinesis failure triggers hippo tumor suppressor pathway activation. Cell. 2014; 158:833-848.
21. Niu ZS, Niu XJ, Wang WH. Genetic alterations in hepatocellular carcinoma: An update. World J

Gastroenterol. 2016; 22:9069-9095.
22. Liu YJ, Zhou Y, Yeh MM. Recurrent genetic alterations in hepatitis C-Associated hepatocellular carcinoma detected by genomic microarray: a genetic, clinical and pathological correlation study. Mol Cytogenet. 2014; 7:81.
23. Hyeon J, Ahn S, Park CK. CHD1L is a marker for poor prognosis of hepatocellular carcinoma after surgical resection. Korean J Pathol. 2013; 47:9-15.
24. Pedica F, Ruzzenente A, Bagante F, Capelli P, Cataldo I, Pedron S, Iacono C, Chilosi M, Scarpa A, Brunelli M, Tomezzoli A, Martignoni G, Guglielmi A. A re-emerging marker for prognosis in hepatocellular carcinoma: the add-value of fishing c-myc gene for early relapse. PLoS One. 2013; 8:e68203.
25. Guido M, Fassan M, Giacomelli L, Cillo U, Farinati F, Burra P, Fagiuoli S, Rugge M. Micronuclei and broken eggs in human liver carcinogenesis. Anticancer Res. 2008; 28:2507-2511.
26. Stephens PJ, Greenman CD, Fu B, *et al*. Massive genomic rearrangement acquired in a single catastrophic event during cancer development. Cell. 2011; 144:27-40.
27. Cortés-Ciriano I, Lee JJ, Xi R, Jain D, Jung YL, Yang L, Gordenin D, Klimczak LJ, Zhang CZ, Pellman DS, PCAWG Structural Variation Working Group, Park PJ; PCAWG Consortium. Comprehensive analysis of chromothripsis in 2,658 human cancers using whole-genome sequencing. Nat Genet. 2020; 52:331-341.
28. Pezzuto F, Izzo F, Buonaguro L, Annunziata C, Tatangelo F, Botti G, Buonaguro FM, Tornesello ML. Tumor specific mutations in TERT promoter and CTNNB1 gene in hepatitis B and hepatitis C related hepatocellular carcinoma. Oncotarget. 2016; 7:54253-54262.
29. Zucman-Rossi J, Villanueva A, Nault JC, Llovet JM. Genetic Landscape and Biomarkers of Hepatocellular Carcinoma. Gastroenterology. 2015; 149:1226-1239.e4.
30. Schulze K, Imbeaud S, Letouzé E, *et al*. Exome sequencing of hepatocellular carcinomas identifies new mutational signatures and potential therapeutic targets. Nat Genet. 2015; 47:505-511.
31. Sung WK, Zheng H, Li S, *et al*. Genome-wide survey of recurrent HBV integration in hepatocellular carcinoma. Nat Genet. 2012; 44:765-769.
32. Nault JC, Calderaro J, Di Tommaso L, Balabaud C, Zafrani ES, Bioulac-Sage P, Roncalli M, Zucman-Rossi J. Telomerase reverse transcriptase promoter mutation is an early somatic genetic alteration in the transformation of premalignant nodules in hepatocellular carcinoma on cirrhosis. Hepatology. 2014; 60:1983-1992.
33. Pilati C, Letouzé E, Nault JC, *et al*. Genomic profiling of hepatocellular adenomas reveals recurrent FRK-activating mutations and the mechanisms of malignant transformation. Cancer Cell. 2014; 25:428-441.
34. Park JI, Venteicher AS, Hong JY, Choi J, Jun S, Shkreli M, Chang W, Meng Z, Cheung P, Ji H, McLaughlin M, Veenstra TD, Nusse R, McCrea PD, Artandi SE. Telomerase modulates Wnt signalling by association with target gene chromatin. Nature. 2009; 460:66-72.
35. Ghosh A, Saginc G, Leow SC, Khattar E, Shin EM, Yan TD, Wong M, Zhang Z, Li G, Sung WK, Zhou J, Chng WJ, Li S, Liu E, Tergaonkar V. Telomerase directly regulates NF-κB-dependent transcription. Nat Cell Biol. 2012; 14:1270-1281.
36. Zhan T, Rindtorff N, Boutros M. Wnt signaling in cancer. Oncogene. 2017; 36:1461-1473.
37. Spranger S, Bao R, Gajewski TF. Melanoma-intrinsic β-catenin signalling prevents anti-tumour immunity. Nature. 2015; 523:231-235.
38. Pinyol R, Sia D, Llovet JM. Immune exclusion-Wnt/CTNNB1 class predicts resistance to immunotherapies in HCC. Clin Cancer Res. 2019; 25:2021-2023.
39. Guichard C, Amaddeo G, Imbeaud S, *et al*. Integrated analysis of somatic mutations and focal copy-number changes identifies key genes and pathways in hepatocellular carcinoma. Nat Genet. 2012; 44:694-698.
40. Chiang DY, Villanueva A, Hoshida Y, *et al*. Focal gains of VEGFA and molecular classification of hepatocellular carcinoma. Cancer Res. 2008; 68:6779-6788.
41. Llovet JM. Focal gains of VEGFA: candidate predictors of sorafenib response in hepatocellular carcinoma. Cancer Cell. 2014; 25:560-562.
42. Calvisi DF, Ladu S, Gorden A, Farina M, Conner EA, Lee JS, Factor VM, Thorgeirsson SS. Ubiquitous activation of Ras and Jak/Stat pathways in human HCC. Gastroenterology. 2006; 130:1117-1128.
43. Newell P, Toffanin S, Villanueva A, *et al*. Ras pathway activation in hepatocellular carcinoma and anti-tumoral effect of combined sorafenib and rapamycin *in vivo*. J Hepatol. 2009; 51:725-733.
44. Villanueva A, Chiang DY, Newell P, *et al*. Pivotal role of mTOR signaling in hepatocellular carcinoma. Gastroenterology. 2008; 135:1972-1983.
45. Tovar V, Alsinet C, Villanueva A, *et al*. IGF activation in a molecular subclass of hepatocellular carcinoma and pre-clinical efficacy of IGF-1R blockage. J Hepatol. 2010; 52:550-559.
46. Totoki Y, Tatsuno K, Covington KR, *et al*. Trans-ancestry mutational landscape of hepatocellular carcinoma genomes. Nat Genet. 2014; 46:1267-1273.
47. Letouzé E, Shinde J, Renault V, *et al*. Mutational signatures reveal the dynamic interplay of risk factors and cellular processes during liver tumorigenesis. Nat Commun. 2017; 8:1315.
48. Nault JC, De Reyniès A, Villanueva A, *et al*. A hepatocellular carcinoma 5-gene score associated with survival of patients after liver resection. Gastroenterology. 2013; 145:176-187.
49. Hoshida Y, Villanueva A, Kobayashi M, *et al*. Gene expression in fixed tissues and outcome in hepatocellular carcinoma. N Engl J Med. 2008; 359:1995-2004.
50. Yamashita T, Forgues M, Wang W, Kim JW, Ye Q, Jia H, Budhu A, Zanetti KA, Chen Y, Qin LX, Tang ZY, Wang XW. EpCAM and alpha-fetoprotein expression defines novel prognostic subtypes of hepatocellular carcinoma. Cancer Res. 2008; 68:1451-1461.
51. Ji J, Yamashita T, Budhu A, *et al*. Identification of microRNA-181 by genome-wide screening as a critical player in EpCAM - positive hepatic cancer stem cells. Hepatology. 2009; 50:472-480.
52. Budhu A, Jia HL, Forgues M, Liu CG, Goldstein D, Lam A, Zanetti KA, Ye QH, Qin LX, Croce CM, Tang ZY, Wang XW. Identification of metastasis-related microRNAs in hepatocellular carcinoma. Hepatology. 2008; 47:897-907.
53. Villanueva A, Portela A, Sayols S, *et al*. DNA methylation-based prognosis and epidrivers in hepatocellular carcinoma. Hepatology. 2015; 61:1945-1956.
54. Jusakul A, Cutcutache I, Yong CH, *et al*. Whole-genome and epigenomic landscapes of etiologically distinct subtypes of cholangiocarcinoma. Cancer Discov. 2017; 7:1116-1135.
55. Fujimoto A, Furuta M, Shiraishi Y, *et al*. Whole-genome

mutational landscape of liver cancers displaying biliary phenotype reveals hepatitis impact and molecular diversity. Nat Commun. 2015; 6:6120.
56. Nakamura H, Arai Y, Totoki Y, *et al*. Genomic spectra of biliary tract cancer. Nat Genet. 2015; 47:1003-1010.
57. Zheng H, Pomyen Y, Hernandez MO, *et al*. Single-cell analysis reveals cancer stem cell heterogeneity in hepatocellular carcinoma. Hepatology. 2018; 68:127-140.
58. Candia J, Bayarsaikhan E, Tandon M, Budhu A, Forgues M, Tovuu LO, Tudev U, Lack J, Chao A, Chinburen J, Wang XW. The genomic landscape of Mongolian hepatocellular carcinoma. Nat Commun. 2020; 11:4383.
59. Takeda H, Takai A, Kumagai K, Iguchi E, Arasawa S, Eso Y, Shimizu T, Ueda Y, Taura K, Uemoto S, Kita R, Haga H, Marusawa H, Fujimoto A, Seno H. Multiregional whole-genome sequencing of hepatocellular carcinoma with nodule-in-nodule appearance reveals stepwise cancer evolution. J Pathol. 2020; doi: 10.1002/path.5533. Online ahead of print.
60. Tsimberidou AM. Targeted therapy in cancer. Cancer Chemother Pharmacol. 2015; 76:1113-1132.
61. Llovet J, Ricci S, Mazzaferro V, *et al*. Sorafenib in advanced hepatocellular carcinoma. N Engl J Med. 2008; 359:378-390.
62. Cheng AL, Kang YK, Chen Z, *et al*. Efficacy and safety of sorafenib in patients in the Asia-Pacific region with advanced hepatocellular carcinoma: a phase III randomised, double-blind, placebo-controlled trial. Lancet Oncol. 2009; 10:25-34.
63. Couri T, Pillai A. Goals and targets for personalized therapy for HCC. Hepatol Int. 2019; 13:125-137.
64. Pinyol R, Montal R, Bassaganyas L, *et al*. Molecular predictors of prevention of recurrence in HCC with sorafenib as adjuvant treatment and prognostic factors in the phase 3 STORM trial. Gut. 2019; 68:1065-1075.
65. Horwitz E, Stein I, Andreozzi M, *et al*. Human and mouse VEGFA-amplified hepatocellular carcinomas are highly sensitive to Sorafenib treatment. Cancer Discov. 2014; 4:730-743.
66. Jiang Y, Sun A, Zhao Y, *et al*. Proteomics identifies new therapeutic targets of early-stage hepatocellular carcinoma. Nature. 2019; 567:257-261.
67. Bruix J, Qin S, Merle P, *et al*. Regorafenib for patients with hepatocellular carcinoma who progressed on sorafenib treatment (RESORCE): a randomised, double-blind, placebo-controlled, phase 3 trial. Lancet. 2017; 389:56-66.
68. Kudo M, Finn RS, Qin S, *et al*. Lenvatinib versus sorafenib in first-line treatment of patients with unresectable hepatocellular carcinoma: a randomised phase 3 non-inferiority trial. Lancet. 2018; 391:1163-1173.
69. Zhu AX, Kang YK, Yen CJ, *et al*. Ramucirumab after sorafenib in patients with advanced hepatocellular carcinoma and increased α-fetoprotein concentrations (REACH-2): a randomised, double-blind, placebo-controlled, phase 3 trial. Lancet Oncol. 2019; 20:282-296.
70. Abou-Alfa GK, Meyer T, Cheng AL, *et al*. Cabozantinib in patients with advanced and progressing hepatocellular carcinoma. N Engl J Med. 2018; 379:54-63.
71. Lee Y-S, Kim BH, Kim BC, Shin A, Kim JS, Hong SH, Hwang JA, Lee JA, Nam S, Lee SH, Bhak J, Park JW. SLC15A2 genomic variation is associated with the extraordinary response of sorafenib treatment: whole-genome analysis in patients with hepatocellular carcinoma. Oncotarget. 2015; 6:16449-16460.
72. Tandia M, Mhiri A, Paule B, Saffroy R, Cailliez V, Noé G, Farinotti R, Bonhomme-Faivre L. Correlation between clinical response to sorafenib in hepatocellular carcinoma treatment and polymorphisms of P-glycoprotein (ABCB1) and of breast cancer resistance protein (ABCG2): monocentric study. Cancer Chemother Pharmacol. 2017; 79:759-766.
73. Wei D, Zhang H, Peng R, Huang C, Bai R. ABCC2 (1249G>A) polymorphism implicates altered transport activity for sorafenib. Xenobiotica. 2017; 47:1008-1014.
74. Lian G, Yuan J, Gao Y. *In vitro* transport ability of ABCC2 (G1249A) polymorphic variant towards anticancer drugs. Onco Targets Ther. 2020; 13:1413-1419.
75. Schaller L, Lauschke VM. The genetic landscape of the human solute carrier (SLC) transporter superfamily. Hum Genet. 2019; 138:1359-1377.
76. Cabral LKD, Tiribelli C, Sukowati CHC. Sorafenib resistance in hepatocellular carcinoma: the relevance of genetic heterogeneity. Cancers (Basel). 2020; 12:1576.
77. Finn RS, Qin S, Ikeda M, *et al*. Atezolizumab plus bevacizumab in unresectable hepatocellular carcinoma. N Engl J Med. 2020; 382:1894-1905.
78. Davoli T, Uno H, Wooten EC, Elledge SJ. Tumor aneuploidy correlates with markers of immune evasion and with reduced response to immunotherapy. Science. 2017; 355: eaaf8399.
79. Bassaganyas L, Pinyol R, Esteban-Fabró R, Torrens L, Torrecilla S, Willoughby CE, Franch-Expósito S, Vila-Casadesús M, Salaverria I, Montal R, Mazzaferro V, Camps J, Sia D, Llovet JM. Copy number alteration burden differentially impacts immune profiles and molecular features of hepatocellular carcinoma. Clin Cancer Res. 2020; doi: 10.1158/1078-0432.CCR-20-1497. Online ahead of print.
80. Lu L, Jiang J, Zhan M, Zhang H, Wang QT, Sun SN, Guo XK, Yin H, Wei Y, Li SY, Liu JO, Li Y, He YW. Targeting Tumor-Associated Antigens in Hepatocellular Carcinoma for Immunotherapy: Past Pitfalls and Future Strategies. Hepatology. 2020; doi: 10.1002/hep.31502. Online ahead of print.
81. Banini BA, Sanyal AJ. The use of cell free DNA in the diagnosis of HCC. Hepatoma Res. 2019; 5:34.

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