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Cellular heterogeneity and plasticity in liver cancer

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ABSTRACT

Hepatocarcinogenesis involves complex genetic and cellular dysregulations which drive the formation of hepatocellular carcinoma (HCC), the predominant form of primary liver cancer, with extensive heterogeneity. In contrast to the broad spectrum of molecularly driven therapies available for defined patient groups in certain cancer types, unfortunately the treatment options for HCC are highly limited. The lack of representative molecular and cellular signatures in the heterogeneous HCC tumors that can effectively guide the choice of the most appropriate treatment among the patients unavoidably limits the treatment outcome. Advancement and wide availability of the next-generation sequencing technologies have empowered us to examine and capture not only the detailed genetic alterations of the HCC cells but also the precise composition of different cell types within the tumor microenvironment and their interactions with the HCC cells at an unprecedented level. The information generated has provided new insight and better defined the inter-patient intertumoral heterogeneity, intra-patient intratumoral heterogeneity as well as the plasticity of HCC cells. These collectively provide a robust scientific basis in guiding the development and use of targeted therapy and immunotherapy. To complement, liquid biopsy coupled with high-sensitivity sequencing could potentially be adopted as a more practical and safer approach to detect and reflect the tumor heterogeneity in HCC patients in guiding the choice of treatment and monitoring disease progression.

1. Introduction

Primary liver cancer is one of the leading cancers worldwide and is aggressive with a high mortality rate. Hepatocellular carcinoma (HCC) and intrahepatic cholangiocarcinoma (iCCA) account for about 85 % and 15 % of primary liver cancer, respectively [1,2]. The median survival rate for patients with advanced HCC could be as short as three months [3]. The currently available treatment options for HCC are limited. Generally, for patients with early and intermediate stage HCCs, surgical resection, liver transplantation, and local ablative therapy are possible curative treatment options. For patients with advanced, inoperable HCC, the first-line systemic therapies with multi-kinase inhibitors including Sorafenib or Lenvatinib have been the standard of care, though their survival benefits are modest and usually accompanied with toxicity and a high chance of developing chemoresistance [4–6]. Besides, current systemic treatment for HCC is given to HCC patients on a 'one-size-fits-all' basis without patient stratification and this limits the effectiveness of treatment and poses an unmet medical need for liver cancer.

2. Tumor heterogeneity of HCC

Tumor heterogeneity refers to the complex cellular composition within a tumor. Throughout carcinogenesis, cancer cells evolve by acquiring multiple genetic and molecular changes that effectively reprogram themselves to exert growth advantages [7]. The reprogramming allows the cancer cells to cope with the cellular stresses due to deprivation of nutrients and oxygen and with the reactive oxygen species that may accumulate and hamper their propagation. As a result, mixed subclones of cancer cells with different genetic and molecular signatures develop, and the heterogeneity of cancer cells within the tumor greatly complicates the choice for the most effective treatment for patients [8]. HCC is characterized by significant inter-tumoral heterogeneity between patients as well as intratumoral heterogeneity within the same tumor of an individual patient [9]. Furthermore, multiple risk factors predispose to HCC development and they include chronic hepatitis B virus (HBV) and hepatitis C virus (HCV) infection, excessive alcohol consumption, nonalcoholic liver disease (NASH), and dietary intake of pro-carcinogenic aflatoxin; they drive HCC initiation and

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progression through different mechanisms [10]. More importantly, the tumors associated with these etiological factors are molecularly distinct and therefore further introduce another layer of heterogeneity to HCC.

As such, the levels of heterogeneity in HCC can be classified into 1) inter-patient inter-tumoral, 2) inter-tumor (multiple nodules in the same liver of individual patient) and 3) intra-tumoral. In addition, tumor heterogeneity can be analyzed from pathology, molecular and genomic angles (Fig. 1).

2.1. Inter-patient inter-tumoral heterogeneity of HCC

2.1.1. Morphological and histological differences

HCC tumors from different patients exhibit profound differences in both morphology and histology. According to the latest (fifth) edition of the WHO classification in 2019, apart from the conventional type, the remaining HCC is classified into histological subtypes with different occurrence rates: steatohepatitic (5–20 %), clear cell (3–7 %), macrotrabecular-massive (5 %), scirrhous (4 %), chromophobe (3 %), fibrolamellar carcinoma (or fibrolamellar HCC, 1 %), neutrophil-rich (<1 %) [11]. Individual subtypes of HCC are associated with a variable set of clinical features and represent different prognostic outcomes. For example, the macrotrabecular-massive subtype, characterized by high serum AFP level, has more frequent vascular invasion, has poor prognosis, and is associated with early and overall recurrence [12]. Furthermore, HCC tumor phenotypes are tightly linked with the underlying molecular changes, and this is often addressed as morpho-molecular subtyping. Recently, a study has examined a large series of HCC and demonstrated that HCCs of different histological subtypes were significantly linked to specific molecular features [13]. Frequent activation of the IL-6/JAK/STAT pathway was observed in the steatohepatitic subtype without alterations in *CTNNB1*, *TERT,* and *p53*; while the scirrhous subtype was mainly associated with the presence of *TSC1/2* mutations, epithelial-to-mesenchymal (EMT) phenotype, and an expression profile more of the liver progenitor cell. In contrast, frequent *TP53* mutation and FGF19 amplification were associated with the more aggressive macrotrabecular-massive subtype.

2.1.2. Molecular classification of HCC: gene expression signatures

The utilization of molecular assays is a more sensitive way to distinguish tumor heterogeneity over the morphologic and immunohistochemical based methods [14]. Thus, the development of a classification system based on the detection of fundamental genetic and molecular changes of HCC would eventually resolve these issues and enhance HCC classification. With transcriptional profiling, multiple research groups have attempted to identify global gene expression signatures in HCCs, categorized them into different molecular subtypes using hierarchical clustering techniques, and subsequently correlated them with their corresponding clinical and phenotypic characteristics [15–19]. So far, HCC could be divided into two molecular classes: 1) the proliferative and 2) non-proliferative classes, as characterized by the enriched mutations and their associated tumor phenotypes [16,17]. In brief, the proliferative class is associated with the enrichment of *p53* mutation, increased chromosome instability, as well as more aggressive and proliferative phenotypes. In contrast, the non-proliferative class is mainly enriched with activation of the Wnt/β-catenin pathway, has a more stable genome, is comparatively less aggressive, and displays more differentiated hepatocytic phenotypes. It has recently been recognized that the non-proliferative subclass HCC is more heterogeneous than originally thought. It could be further subclassified into periportal and perivenous subclasses according to their intrinsic metabolic zonation programs that govern their original metabolic functions along the porto-central axis in the normal liver [20]. Genetically, periportal and perivenous subclasses have a background of wildtype and mutant β-catenin, respectively, and different hepatic metabolic functions. However, they express negatively correlated gene networks that are mutually exclusive to one other. In particular, the periportal subclass mainly expresses a *HNF4A*-driven gene signature and represents a group of HCC with a low potential of early recurrence and highest survival rate.

Besides establishing the gene signatures at transcriptional level, the exploration of the molecular classification of HCC has also been extended to proteomic levels. Jiang et al. carried out a label-free, mass spectrometry-based proteomic analysis in over a hundred pairs of early-

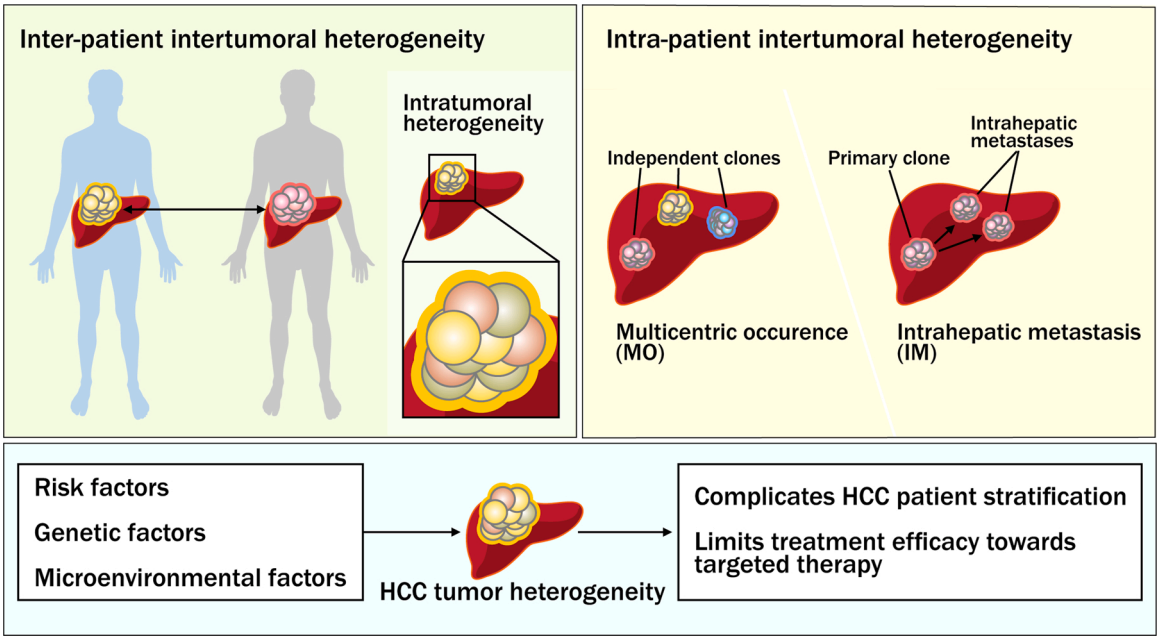

Fig. 1. HCC heterogeneity and its translational implication. HCC heterogeneity can be classified into 1) inter-patient intertumoral heterogeneity, 2) intra-patient intertumoral heterogeneity (multiple nodules in the same liver of individual patient) and 3) intratumoral heterogeneity. Multiple tumor nodules in the liver can arise from genetically independent clones, also known as multicentric occurrence (MO), or originate and be derived from a primary clone by intrahepatic metastasis (IM). Multiple factors are interconnected and contribute to the HCC tumor heterogeneity. The extensive heterogeneity of the HCC tumor complicates HCC patient stratification and limits treatment efficacy towards targeted therapy.

staged HBV-associated HCC patient samples and successfully captured an average of over 5000 proteins in both the tumor and control tissues. By non-negative matrix factorization consensus clustering (NMF), the HCC tumors were stratified into three proteomic subtypes, known as SI, SII, and SIII, with an ascending order of tumor aggressiveness [21]. The least aggressive SI HCC proteomic subtype resembled the non-proliferative class of HCC previously described, showed an upregulation of liver metabolic proteins and had a better prognosis. In contrast, the SII and SIII subtypes, which expressed higher levels of proteins that support proliferative functions and increased metabolic adaptation towards glycolysis and cholesterol metabolism, were more aggressive and had a worse prognosis, as in the proliferative class. Though there is a continuous momentum in developing the molecular signature with different approaches, clinical application of the currently proposed molecular signatures of HCC has not been widely adopted. This is possibly due to the classification discrepancies, the unaddressed translational value of these signatures, the unmet requirement of the sophisticated testing platform, and limited scalability in the actual clinical setting.

2.1.3. Molecular classification of HCC: gene mutations

Searching for actionable driver mutations for designing promising therapy has long been set as one of the top priorities in liver cancer research. In the last decade, the availability and technical advancement in next-generation sequencing (NGS) have enabled us to examine the genetic, epigenetic, and transcriptomic changes in cohorts of bulk HCC tissues at an unprecedented level. The unbiased, global genomic study has led us to appreciate HCC as a cancer type that lacks a representative driver mutation and is predominantly driven by recurrent loss-of-function mutations in tumor suppressor genes including *P53*, *AXIN1*, *ARID1A* and *TSC1/2* [22–24]. Although oncogenic mutations at *TERT* promoter and *CTNNB1* have also been recurrently found in a subset of patients, providing significant biological insight into HCC development and molecular classification, these targets are regarded as undruggable and their translational potential is still questionable. Of note, different sets of private mutations or less abundant mutations are usually observed in tumors from different patients, indicating substantial inter-patient intertumoral heterogeneity in HCC. However, the approach of sequencing bulk HCC tumors sometimes refrains us from understanding whether these mutations ubiquitously affect all the tumor cells or are only present in confined regions within those tumors. Also, there is query whether a single biopsy of the tumor tissues at the time of surgical resection is sufficient for deducing the chronological order of the genetic aberrations throughout the HCC development.

2.2. Intra-patient inter-tumoral heterogeneity

Multinodular HCCs can develop in the liver simultaneously, and this poses an important biological question whether they arise from the same primary tumor which has disseminated through intrahepatic metastasis (IM) or whether they develop independently as multicentric occurrence (MO). These underlying mechanistic differences have significantly different prognostication. The clonality of the individual tumors within the same patients can be used to distinguish these two categories, with monoclonality reflecting the multiple tumors arising from a common lesion and therefore IM, and disparate clonality suggesting that the multiple tumors are not derived from a common origin and therefore mulitcentric origin (MO). Based on the experience of our center, examination of the clonality by an array of molecular assays including DNA aberrations and HBV viral integration in these tumors revealed a ~4:6 ratio between the MO and IM in a cohort of HBV-associated HCC patients [25]. With the technological advancement in sequencing technologies, the molecular characteristics between the IM and MO multinodular tumors have been revisited [26–30]. Using powerful whole-genome and RNA sequencing, it was consistently demonstrated that MO tumors showed a much higher degree of heterogeneity than the IM tumors, in terms of structural variations, copy number alterations, and the variant allele frequencies at the genome levels. Furthermore, IM tumors tended to genetically evolve in a divergent manner upon continuous systemic treatment with Sorafenib [27]. The continuous evolvement of IM tumors, together with the low incidence of amplification of sorafenib-target genes in the multifocal tumors [29], may partly provide a plausible explanation why a low response rate and sorafenib insensitivity are commonly observed in patients with multinodular HCC tumors.

HCC tumors can also arise spatio-temporally that new tumors form in the remnant liver after hepatic resection. A 2-year cut-off has been proposed to distinguish between IM tumors (early recurrence event) and de novo MO tumors (late recurrence event) [31,32]. Though technically challenging and restricted by the availability of biopsy tissues, multiple sampling of HCC tumor tissues at 1) multiple sites within a single HCC tumor as well as the intrahepatic tissues, or, at 2) different time points in the same individual followed by systematic analysis by high-resolution sequencing would provide us valuable, multi-dimensional information regarding the spatio-temporal heterogeneity as well as the progression of HCC tumor.

Xue et al. comprehensively examined the genetic changes of tumor evolution from primary HCC tumors to local intrahepatic metastasis [26]. They systematically subjected a total number of 43 samples isolated from 10 HBV-associated HCC tumors, including primary HCC tumors, their intrahepatic metastases (IM), satellite nodules (SN), and tumor thrombi (TT), to multiregional exome- and low-coverage whole DNA sequencing analyses. In brief, IM showed a variable degree of heterogeneity with the primary HCC, suggesting that IMs can occur early or late during tumor progression. Interestingly, SN shared 90 % of mutations in the primary tumor, suggesting that SN was derived from the late seeding of tumor cells originated from the primary tumors. The majority (8/11) of the TTs shared more than 90 % of nonsynonymous mutations with their primary matches. Upon correlation of the intratumoral heterogeneity of different lesions with tumor size, it was found that patients who had larger tumor size had significantly higher degree of intratumoral heterogeneity. This further suggests that local metastases in smaller tumors likely are more homogeneous, while local metastases in larger tumors exhibit a comparatively increased heterogeneity.

To examine and pinpoint the key genomic features in clonal evolution and tumor relapse, Ding et al. carried out multiregional next-generation DNA sequencing in 113 patients of 365 primary and their recurrent HCC tumor samples [33]. A complementary DNA methylation profiling was also included in the majority of the patient samples to reveal the epigenomic events in both the tumors and non-tumorous tissues. They reconfirmed that the critical HCC driver mutations including *TERT*, *p53*, and *CTNNB1* were shared among HCC tumors, suggesting that they are key early genetic events in HCC development. Interestingly, intratumoral heterogeneity was also observed at the epigenetic levels in terms of aberrant DNA methylation. Importantly, they suggested that the epigenetic changes were critical, occurred as early as during fibrosis or cirrhosis before the early key genetic events, and exerted a “field effect” which predisposed the liver to the tumor development. These findings emphasized the significant interplay between the genetic and epigenetic alterations at different stages of HCC development contributing to tumor heterogeneity.

2.3. Intra-tumoral heterogeneity of HCC

Trunk mutation refers to those genetic changes, usually affecting the coding region and ubiquitously shared among all the tumor cells within a tumor from the same origin, while branch mutations are usually carried by a subset of tumor cells which clonally evolve from the original tumor. By first assuming those mutations present in dysplastic nodules and small HCC as trunk mutations, Torrecilla et al. examined the preservation of these mutations (eventually defined as bona fide trunk mutations, including *TERT* promoter mutation, *p53,* and *CTNNB1*

mutations) in different regions within the large tumors [34]. It was found that these molecular changes were well conserved in the large tumors and could be passed onto more than 85 % of the metastatic tissues. A similar architecture of genetic lineage has also been suggested in another study [35]. Similarly, substantial intra-tumoral heterogeneity was observed from neoantigen and HBV antigen analyses. DNA-based tumor clonality was found to out-perform the number of DNA mutations in predicting the overall survival of HCC patients. Intra-tumoral heterogeneity signature of 363 genes was derived from differential expression analysis of multi-regional samples and such signature was found to be associated with poorer overall survival. Collectively, the findings highlight the possible role of intra-tumoral heterogeneity in predicting HCC prognosis. Though HCC exhibits extensive intratumoral heterogeneity, these findings however further suggest that a single tumor biopsy is sufficient to capture the trunk mutations in HCC patients, and is informative in terms of assigning patients to different molecular subclasses. An independent study has also echoed and demonstrated that a single sampling should be sufficient to capture more than 90 % of the mutations within the whole HCC tumor with monoclonal origin [33]. These findings give assurance that studying HCC patient samples using single sampling does reflect a certain intratumoral characteristic of the whole tumor. However, the most optimal number of biopsies to be included remains to be systematically explored with HCC tumors which have different clinicopathological features such as different tumor sizes or metastatic potential. However, the biopsy issue of HCC for treatment is challenging, with the risk of bleeding and potential tumor seedling.

2.3.1. The whole catalog of liver cells

Liver is a critical metabolic hub in the body and its functions are accomplished by the orchestration of multiple cell types in a highly coordinated manner. Recently, powerful single-cell RNA sequencing (scRNA-seq) has been adopted to profile all the normal cell types in the liver and generate an array of expression profiles which act like molecular prints of their unique identities [36]. A recent study has sequenced 10,000 single cells from nine normal liver samples from patients, identifying the different types of cells in the normal human liver. This generation of a complete liver atlas serves as a valuable reference facilitating the cell type identification and comparison in the liver at the molecular level. For example, it enables the identification of a new population of bipotent liver progenitor cells expressing EpCAM, a hepatic stem cell marker, which is distinct from the hepatocyte-biased and cholangiocyte populations as compared to the liver atlas. This new bipotent liver progenitor, through modulating its TROP2 expression, is able to differentiate into either cholangiocytic or hepatocytic lineages and plays a critical role during self-regeneration upon liver tissue damage.

2.3.2. Bipotent liver progenitor cells and primary liver cancer

In addition to playing a normal physiological function during hepatic development and tissue repair upon injury, bipotent liver progenitor cells could contribute to the tumor heterogeneity of HCC and iCCA [37]. Traditionally, multiregional sampling was utilized to investigate intratumoral heterogeneity of iCCA [38]. Recently, with the use of genomic, transcriptomic, and single nucleus sequencing, the clonality of the separate, mixed, and combined HCC−CCA was examined at high resolution [39]. Mixed and combined subtypes were two distinct tumor subtypes that molecularly resemble the HCC and CCA, respectively; thus corresponding treatment that more preferentially targets HCC or CCA should be provided to different subsets of HCC−CCA patients accordingly. Interestingly, combined and mixed HCC−CCA subtypes were both demonstrated to be of monoclonal origin. The cell fate of the primary liver cancer cells and cells in HCC−CCA tumors may also exert robust cellular plasticity in response to the hepatic tumor microenvironment. In another scRNA-seq study of HCC and CCA patients, the intratumoral heterogeneity was estimated based on the diversity score of tumor cells. Patients with higher tumor diversity score demonstrated significantly poorer overall and progression-free survivals, indicating higher level of transcriptomic diversity could lead to more aggressive tumor characteristics. Interesting, the diversity scores for CCA cases were significantly higher than that of HCC cases. There was a significant trend for association between transcriptomic diversity and overall survival. The genomic diversity estimated based on inferred copy number variation was similarly associated with transcriptomic diversity and prognosis [40].

2.3.3. Heterogeneity of liver cancer stem cells

It has been suggested that the introduction of abnormal genetic changes in the bipotent liver progenitor cells or dedifferentiation of the HCC cells generates a liver progenitor cell-like population known as liver cancer stem cells (CSCs). Liver CSCs have the ability to self-renew and differentiate into a heterogeneous population of tumor cells to constitute a tumor following a hierarchical structure. HCC cells that possess CSC properties are crucial in driving tumor initiation, supporting aggressive tumor behavior, leading to the resistance to treatment and tumor relapse [41,42]. Multiple liver CSC markers including EpCAM, CD133, CD90, CD44, CD13, CD24, and CD47 have been identified to be associated with liver cancer stemness under different biological contexts [43–49]. However, a comprehensive evaluation of the heterogeneity of the liver CSC population has not been systematically carried out until recently.

Zheng et al. performed scRNA-seq to evaluate the liver CSC heterogeneity by simultaneously examining the proportion of CD133+, EpCAM+, CD24+, and triple marker positive or negative cell populations in two different HCC cell lines, Huh1 and Huh7, and one primary HCC tissue [50]. Consistently, great inter-tumoral heterogeneity existed among these three samples. Examination within individual samples showed that HCC cells with specific CSC marker expression clustered together, shared a similar gene signature, and were associated with specific molecular pathways. For example, CD133+ cells and EpCAM + cells were associated with Akt and p38MAPK pathways, while CD24+ or CD133, EpCAM, and CD24 triple-positive cells are associated with NF-kB pathway. The study also found that CSC marker expression could predict prognosis; HCC patients having triple marker-positive expression showed a significantly lower overall survival than those with triple marker-negative expression. More importantly, a heterogeneity-surrogate gene signature, derived from a set of genes that correlated with all the CSC marker expressions, was suggested to be a prognostic indicator to independently predict the survival outcome of HCC patients. Taken together, liver CSCs are highly heterogeneous in terms of their phenotypes. The heterogeneity and relative abundance of different CSC populations in the HCC tumor may affect the tumor progression and outcome of patients.

Besides, scRNA-seq has been used to identify rare CSC subpopulations in HCC. In a study examining a single case of patient-derived HCC tumor xenograft, 139 HCC cells were successfully captured and sequenced by scRNA-seq, and two cell populations showing differentially EpCAM expressions were identified. Further examination of the expression of an array of liver CSC markers in the EpCAM positive population led to the identification of a new stemness-related subclone represented by dual CD24 and CD44 positivity. These EpCAM+/CD24+/CD44+ subclones showed a signature gene expression pattern, including the upregulation of hypervariable genes including S100A, VIM, CD44, CTSE, and KRT20, which are potentially critical in supporting cancer stemness properties. As a proof of concept, shRNA-mediated CTSE knockdown in CD24+/CD44 + HCC cells significantly attenuated their self-renewal ability and in vivo tumorigenicity [51]. The findings further supported the notion that identification of and targeting specific CSC cell populations among the heterogeneous cell population within the HCC the tumor may have therapeutic implications.

3. Immune cell heterogeneity in HCC

Liver functions as an important site for innate immunity as it keeps being exposed to a massive amount of antigens and metabolites generated by food intake as well as gut biota that are constantly transported to the liver through the portal vein from the gastrointestinal tract [52]. Kupffer cells, a specialized type of macrophage residing in the liver, serve as the first-line defence to detect and clear away the foreign pathogens from the circulation across the liver. Also, the Kupffer cells together with the liver sinusoidal endothelial cells gear up with functional antigen-presenting machinery that can interact and activate effector T-lymphocytes. It is recognized that the liver microenvironment is comparatively immunotolerant to avoid unnecessary immune responses targeting non-pathogenic antigens. Similarly, in livers with chronic inflammation and viral hepatitis infection, the sustained activation of the immune system can also lead to immune exhaustion, eventually shaping a more immunosuppressive microenvironment. As immune evasion is one of the hallmarks of cancer, the immunosuppressive microenvironment in the liver would probably serve as a strong pro-oncogenic factor in supporting HCC initiation and progression.

Harnessing the immune system is a revolutionary approach in treating cancers including HCC. Several immune checkpoint inhibitors (ICI) including anti-PD1 antibodies Nivolumab and Pembrolizumab have been approved as the second-line agents for treating advanced HCC patients who are refractory to the Sorafenib treatment [53,54]. Until very recently, a combo treatment of Atezolizumab (anti-PD-L1 antibody) plus Bevacizumab (anti-VEGF antibody) has been demonstrated to exhibit an overall better survival benefit than Sorafenib in a randomized Phase III clinical trial in a first-line setting [55]. These encouraging data support the efficacy of immunotherapy for HCC treatment. Although some HCC patients show a complete or partial response to the ICI with comparatively less side effects as compared to molecular targeted drugs, the general overall response rate still remains unsatisfactory (less than 20 % for Nivolumab) [53]. In recent years, multiple studies have examined the immune cell landscape and its heterogeneity in HCC. These explorations may help define the immune tumor microenvironment in HCC, which can serve as an important scientific basis for identifying patients who can be benefited from immunotherapy. They may also reveal additional immune checkpoint molecules and related mechanisms that can be harnessed for improving the outcome of the currently available immunotherapy.

3.1. Immune gene signature in HCC

Tumors from different patients show a different degree of immune cell infiltration and immune cell composition. Immunologically hot tumors showing high levels of immune cell infiltration of T cells are more susceptible to ICI treatment when compared to immunologically cold tumors. In order to establish a classification system that can categorize HCC patients into different immune subclasses, multiple studies have examined the immune cell composition in HCC tumors, followed by correlating the observed immune characteristics to specific pathological and molecular features [56–58] (Fig. 2A).

Inflammatory response is usually an indication of the positive immune response. By deconvolution of RNA-expression data of 228 HCC samples as a training set into tumor, stromal and immune cell components, Sia et al. examined the gene expression pattern of inflammatory cells and cross-confirmed the presence of immune infiltrates and marker expression by histopathological and immunohistochemistry (IHC) analyses. These analyses yielded a new class of HCC patients known as Immune class [56]. Immune-class HCC was characterized by the presence of expression signatures of CD4 helper and CD8 cytotoxic lymphocytes, the appearance of high level of immune cell infiltration, and the positive expression of immune checkpoint marker proteins including

A

HCC Immune signatures

Sia et al. 2017	Immune class: Active immune		Immune class: Exhausted immune
	T cell receptor, CD8A, IFNγ and Granzyme B expression		Activation of TGFβ signaling, Increased M2 macrophage
	Associated with Non-proliferation class HCC		Associated with proliferation class HCC
	Better prognosis		Comparatively worse prognosis
Kurebayashi et al. 2018	Immune high	Immune mid	Immune low
	Increased B cell and T cell infiltration	Moderately Increased T cell and reduced B cell infiltration	Low immune cell infiltration
	Associated with poorly differentiated HCC and proliferation class HCC	Associated with fibrosis	
	Better prognosis	Comparatively worse prognosis	
Zhang et al. 2019	Immuno-competent	Immuno-deficient	Immuno-suppression
	Normal T cell infiltration and lower Breg cells	Reduced lymphocyte infiltration, increased dendritic cells and NK cells	Increased Treg cells, Breg cells and M2 macrophages Increased PD-1. PD-L1, Tim-3 and CTLA4 expression
	Enhanced expression of Pro-T cell recruitment cytokines including CXCL9, CXCL10, CXCL11, CXCL16	Restricted expression of monocyte and macrophage recruitment cytokine CCL14	Increased VEGFA expression and immunosuppressive TGFβ,CCL8 and IL10
	Better prognosis	Comparatively worse prognosis	

B

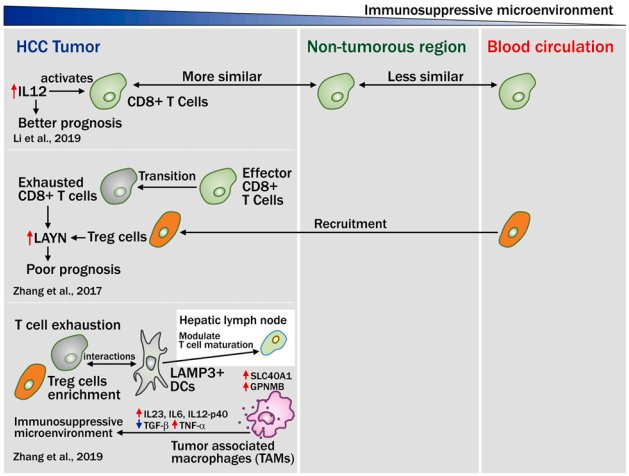

Fig. 2. Immune cell heterogeneity in HCC. (A) A summary of the representative immune gene signatures in human HCC. Different immune subclasses or subtypes are defined to categorize HCC patients into different subgroups with distinct tumor immune characteristics and prognostic implications. (B) Schematic diagram illustrating the heterogeneity of T lymphocytes and their regulatory cells in HCC. HCC tumors exhibit an immunosuppressive microenvironment when compared to the adjacent non-tumorous livers and the blood circulation. The immunosuppressive HCC tumor microenvironment is shaped by the presence of a specific subset of exhausted CD8 + T cells, which are usually derived from the effector CD8 + T cells, as well as the enrichment of regulatory T cells (Treg cells) which negatively regulate the activity of T cells. Besides, other immune cells such as dendritic cells and tumor-associated macrophages (TAMs) may also directly interact and modulate the T cell functions or secrete proinflammatory cytokines in supporting the creation of the immunosuppressive environment.

PD-1 and PD-L1. More importantly, HCC tumors within the Immune class could be further subdivided into two distinct clusters according to the active (as indicated by the T-cell receptor, CD8A, IFN-γ and Granzyme B expression that mediate immune activation) or exhausted (as indicated by the TGF-β signaling and M2-macrophage that mediate immunosuppression) immune responses in the tumor microenvironment. More importantly, patients with an active immune response likely were associated with a better prognosis when compared with the rest of the patients. Patients of the Immune class signature could also be identified in a validation HCC cohort, TCGA HCC dataset and other publicly available HCC cohorts worldwide, and accounted for around 27 % out of a total 956 HCC patients being examined.

By multiplex IHC analyses for eight different immune cell markers in over 900 multiregional samples of 158 HCC patient samples, Kurebayashi et al. examined and looked for representative immune cells that could be used to predict the prognostic outcome [57]. Based on the immune cells composition, the HCC tumor microenvironment was broadly divided into three subtypes: the 1) Immune high; 2) Immune mid and 3) Immune low subtypes. For the Immune high subtype, it was characterized by an increased B cell and T cell infiltration. Among these cells, the co-infiltration of T and B cells was identified as an independent, positive prognostic factor. Immune high subtype was significantly associated with 20 % of the poorly-differentiated and high-grade HCC, but not the well- to moderately-differentiated HCC. More importantly, high-grade HCC with immune high signature showed a better overall prognosis.

Recently, Zhang et al. carried out CyTOF and multi-omics analysis of 42 multiregional HCC samples from eight patients, enabling a comprehensive clustering analysis of the intratumoral and inter-patient heterogeneity of tumor cells and immune cells in the HCC tumor microenvironment [58]. In general, the degree of immune cell heterogeneity is comparatively lower that of the tumor cells, suggesting that modulating local immunity in the tumor microenvironment might be a better and more universal therapeutic approach for HCC. To examine the immune cell heterogeneity, CyTOF analysis of over four million immune cells identified significant alterations in the immune landscape in HCC and these immune cells could be grouped in forty different cell clusters. Hierarchical clustering of the CyTOF data could categorize the HCC tumor samples into three immuno-types: the Immunocompetent subtype 1 as characterized by the normal T cell infiltration but relatively lower Regulatory B cells (Breg cells); the Immunodeficient subtype 2 as characterized by the reduced lymphocyte infiltration and increased levels of dendritic cells and natural killer cells; and the Immunosuppressive subtype 3 as characterized by the increased population of Regulatory T cells (Treg cells), Breg cells and M2 macrophages together with a higher expression of immunosuppressive checkpoint molecules including PD-1, PD-L1, Tim-3 and CTLA-4. In other words, subtypes 1 and 3 can be regarded as immunologically hot tumors while subtype 2 is immunologically cold. Interestingly, the identified immuno-subtypes well correlated with the tumor metabolic phenotypes. For example, the subtype 1 and subtype 2 showed an increased activity in urea cycle and nucleotide biosynthesis respectively, while subtype 3 exhibited a reduced glycolysis and an enhanced oxidative phosphorylation. The enrichment of specific metabolic pathways among the immune subtypes actually suggested that the end metabolic product generated might also play a critical role in modeling the tumor immune microenvironment. Additionally, expression of different cytokines and chemokines was also associated with the immune subtypes. For instance, the expression of pro-T-cell recruitment molecules including CXCL9, CXCL10, CXCL11, and CXCL16 was enhanced in subtype 1 and subtype 3, while overexpression of VEGFA and some of the immunosuppressive chemokine or cytokine genes including TGFB, CCL8 and IL10 was observed in subtype 3. Comparatively, subtype 2 showed restricted expression of chemokines or cytokines, and only CCL14 which is responsible for recruiting monocytes and macrophage was expressed. Importantly, HCC patients belonging to Immunocompetent subtype 1 showed a significantly better prognosis when compared to other subgroups. Practically, examination of the expression of two markers including CD45 and FoxP3 could potentially be useful to classify the HCC patients into the proposed immune subtypes (Subtype 1: CD45 high/FoxP3 low; Subtype 2: CD45 low/FoxP3 low; Subtype 3: CD45 high/FoxP3 high) and may predict a comparable prognostic outcome. Of note, increased expression of a similar set of immunosuppressive markers has been proposed and demonstrated to depict the increased immunosuppressive HCC tumor microenvironment when compared to the corresponding non-tumorous liver and peripheral blood [59].

3.2. Heterogeneity of T lymphocytes and their regulatory cells in HCC

Besides examining the immune landscape in a global manner, multiple studies have selectively examined specific immune cells isolated from HCC tumors and other relevant immune sites (Fig. 2B). Inactivation of effector T cells and the enrichment of immunosuppressive cells are both important in creating an immunosuppressive HCC tumor microenvironment. However, the key and fundamental question regarding the origin of tumor-specific immune cells in HCC tumors remains to be addressed. Li et al. specifically examined the infiltrated CD8 + T cells in HCC tumors with respect to their corresponding peritumoral regions and peripheral blood mononuclear cells (PBMCs) in eight treatment-naive patients by whole exome and transcriptome sequencing [60]. The tumor infiltrating CD8 + T cells exhibited a higher degree of transcriptional changes than genomic changes. By comparing the gene signatures derived from the transcriptional profiles of the CD8 + T cells at tumoral and peritumoral regions as well as from the peripheral blood, the tumor infiltrating CD8 + T cells were more molecularly alike to the CD8 + T cells in the peritumoral regions than to the peripheral blood. This suggests that a subset of CD8 + T cells might be preferentially enriched to localize within the tumor. Besides, differences in the tumor-associated features might also lead to the heterogeneity of tumor infiltrating CD8 + T cells observed in different HCC patients. Furthermore, a positive CD8 + T cell activity could be reflected by activation of IL-12-mediated pathway in HCC, and HCC patients with a higher IL-12 activation were found to have a better prognosis.

Multiregional tumor sampling was adopted to study the spatiotemporal interactions between cancer and immune cells and intratumoral heterogeneity [61]. Specifically, significant intra-tumoral heterogeneity was observed in terms of the burden of tumor infiltrating lymphocytes. Besides, significant differences in the numbers of unique T cells were also detected in different regions of HCC tumors, suggesting the possibility of local immune clonal expansion. Zheng et al. performed scRNA-seq to capture the expression profiles and T cell receptor (TCR) sequence of more than 5000 single T cells from the peripheral blood, tumor and adjacent non-tumorous liver tissues from six HCC patients [62]. Within the 11 T cell subsets identified, five were enriched with CD8 + T cells (namely cluster C1-C5) while the remaining six were enriched with CD4 + T cells (namely cluster C6-C11). Among these cell clusters, C4 and C5 clusters consisted predominantly of exhausted tumor-associated CD8 + T cells, as characterized by positive CTLA4 and PDCD1 expression, and intermediately exhausted CD8 + T cells with the GZMK gene expression signature. The C7 and C8 clusters had CD4 + Treg cells in the tumor as well as blood with positive FoxP3 expression or other immunosuppressive marker expression such as TNFRSF9, TIGIT and CTLA4. Furthermore, the CD4 + T helper cell population showing exhaustion phenotype as characterized by the expression of immunosuppressive markers including CXCL13, PDCD1, CTLA4, and TIGIT was identified as C10 cluster. Another CD4 + T helper population was grouped as C11 cluster with elevated expression of cytotoxic marker including NKG7, GNLY and GZMB. In HCC, the immunosuppressive Treg cells and exhausted CD8 + T cells were preferentially enriched and clonally expanded. In addition to employing the established immune markers to map the immunosuppressive cells, novel signature gene was also identified. For instance, layilin was found to be a functionally

important gene upregulated in immunosuppressive Treg and exhausted T cells. More importantly, layilin elevation in HCC patients was significantly associated with poor prognosis. One of the highlights of this study is that, based on the TCR sequencing data, they further provided direct evidence on the source of Treg cells and exhausted CD8 + T cells. Specifically, the majority (82 %) of the tumor infiltrating Treg cells identified were directly recruited rather than evolved from T cells residing in the tumor and non-tumorous tissues, as they were found to be genetically unique. In contrast, exhausted CD8 + T cells shared a certain degree of similarities with different CD8 + T cell clusters and were likely to have evolved and transited from effector CD8 + T cells. The blockage of this transition may pose a therapeutic value in sustaining or reactivating the CD8 + T cells in targeting the tumor cells.

Besides Treg cells, additional immune cell types including dendritic cells (DCs) and tumor-associated macrophages (TAMs) have also been functionally implicated in T lymphocyte modulation. The same research group further examined the immune cell landscape of HCC in another study by isolating more than 75,000 CD45+ immune cells from five different immune sites in 16 treatment-naive HCC patients and subjecting them to scRNA-seq analysis [63]. Specifically, they identified a novel population of tumor-associated DCs with positive LAMP3 (LAMP3+ DCs) expression. The group suggested that LAMP3+ DCs were involved in mediating T cell dysfunction in the tumor microenvironment, as their gene expression signatures highly correlated with those of exhausted T cells and Treg cells in HCC. In addition, DCs and T cells showed a significant of immune ligand and receptor-based interactions. By integrating the expression data from multiple immune sites, mature LAMP + DCs possibly migrated from the tumor to the hepatic lymph node, a site where they could prime and modulate T cell maturation. In addition, innate immune cells, especially the TAMs, were enriched in HCC, as characterized by the high expression of SLC40A1, ferroportin and GPNMB, a transmembrane glycoprotein. More importantly, SLC40A1 promoted the production of pro-inflammatory cytokines including IL23, IL6 and IL-12p40 and the suppression of IL1β production; while GPNMB was shown to support tumor necrosis factor alpha (TNF-α) production. These observations suggested that TAMs are important in shaping an immunosuppressive environment and the presence of TAMs was significantly associated with a survival disadvantage in HCC.

3.3. Some considerations for better immunotherapeutic outcome in HCC patients

Although currently available ICI have a well-defined target, however, evaluation of these targets such as PD-L1 in HCC in actual clinical practice is still technically challenging [64]. Also, expression of a single immune checkpoint marker is usually not informative enough to predict the response and outcome of ICI treatment. An effective biomarker in predicting the response of ICI treatment is much awaited. Recently, Galarreta et al. made use of the hydrodynamic tail vein injection model to produce immunogenic liver tumor in mice with defined genetic backgrounds followed by anti-PD1 treatment. Interestingly, they observed that HCC tumors driven by activation of Wnt/β-catenin pathway were non-responsive to anti-PD1 treatment. This was possibly caused by a reduction of CCL5 cytokine in the tumor and eventually abolished the recruitment of critical immune cells including CD103-positive DCs and antigen-specific CD8 + T cells responsible for tumor eradication [65]. These findings are in line with the observation in HCC patients in that HCC tumors carrying gene signatures of Wnt/β-catenin activation are usually excluded from the Immune class [56]. Since a significant proportion of human HCCs is affected by β-catenin activation, screening for β-catenin mutations and/or Wnt pathway dysregulation may serve as potential biomarkers that inform non-responsiveness to immunotherapy. Given that intratumoral immune heterogeneity exists in HCC tumors, the total number of samples needed to be analyzed from a single tumor in order to reliably represent the actual tumor microenvironment remains a critical but also practical question to be answered. Very recently, Shen et al. performed multi-regional IHC for various immune cell markers, PD-L1 and tertiary lymphoid structures in 9 HCC patients to determine the intratumoral immune heterogeneity [66]. Importantly, around 70 % of the tumors exhibited a uniform PD-L1 expression in all regions examined. Besides, the uniformity of the tumor microenvironments in different regions within a tumor was further demonstrated by transcriptomic analysis using RNA-seq. Altogether, these data suggest that a single regional tumor sampling could reliably capture the landscape of the immune microenvironment in the majority of HCC cases.

4. Cellular plasticity in HCC

Plasticity is defined as the ability of a cell to acquire the cell fates or phenotypes of other cell types reversibly in a specific tissue [67]. De-differentiation of differentiated cells to progenitor cells [67] or inter-conversion between different differentiated cell states or phenotypes (trans-differentiation) [67,68] can be considered a plasticity property of the cells. There are certain aspects of plasticity, including epithelial-mesenchymal transition (EMT), differentiation plasticity, and metabolic plasticity [42], and these have been studied in HCC (Fig. 3).

4.1. EMT in HCC

EMT is the transition from epithelial state to the mesenchymal state of the cells. It can be elicited by TGF-β in HCC [69] and fueled by the TGF-β1-CD147 self-sustaining signaling [69] in CD44-positive CSCs. CD147 promotes TGF-β1 expression which also enhances CD147 expression to result in a positive correlation between TGF-β1 and CD147 in the DEN-induced HCC mouse model to drive the HCC cells towards a more mesenchymal state [69].

A study on the circulating tumor cells (CTCs) in HCC patients found that there was a spatial difference in EMT marker expression on the CTCs collected at different locations along the blood flow system, with epithelial CTC released into the hepatic vein from the tumor and gradually mesenchymal CTC along the blood-flow pathway [70]. This may indicate the EMT plasticity of HCC cells may contribute to intra-patient tumor cell heterogeneity.

However, the dual roles of TGF-β as a tumor suppressor and tumor pro-metastatic factor have also been suggested to be stage- and context-dependent in HCC [71–73] and it has been implicated that a context-dependent role of TGF-β on the plasticity of liver cancer cells as compared to normal liver cells exists [71]. For instance, the inhibition of TGF-β confers the plasticity of normal hepatocytes to become bipotent hepatic progenitor cells (HPCs) [74]. Furthermore, in normal liver, the interplay between the TGF-β and HGF/c-Met signaling can drive the EMT and cell expansion of the oval cells, respectively [75]. Upon adaptation to chronic TGF-β exposure, the oval cells are able to alleviate CCl_4-induced liver damage upon transplantation via c-Met signaling-mediated oval cell expansion and promotion of mature hepatocyte phenotype [75]. Further studies are needed to delineate the underlying mechanism for the regulation of EMT plasticity in HCC and normal liver cells.

4.2. Differentiation plasticity in HCC

From a lineage-tracing experiment, both bipotent Sox9+ HPCs and differentiated hepatocytes were found to derive HCC in a galectin-3-dependent manner [76], indicating that the HCC derived from both HPCs and hepatocytes may involve the plasticity across these cell types depending on the context of HCC initiation. Kras and TP53 mutations in Sox9+ cholangiocytes or adult hepatocytes could result in diverse consequences, with the mutation in the former cell type resulting in more profound intrahepatic cholangiocarcinoma (CCA) formation [77]. Interestingly, liver injury by biliary toxin 3,5-diethoxycarbonyl-1,

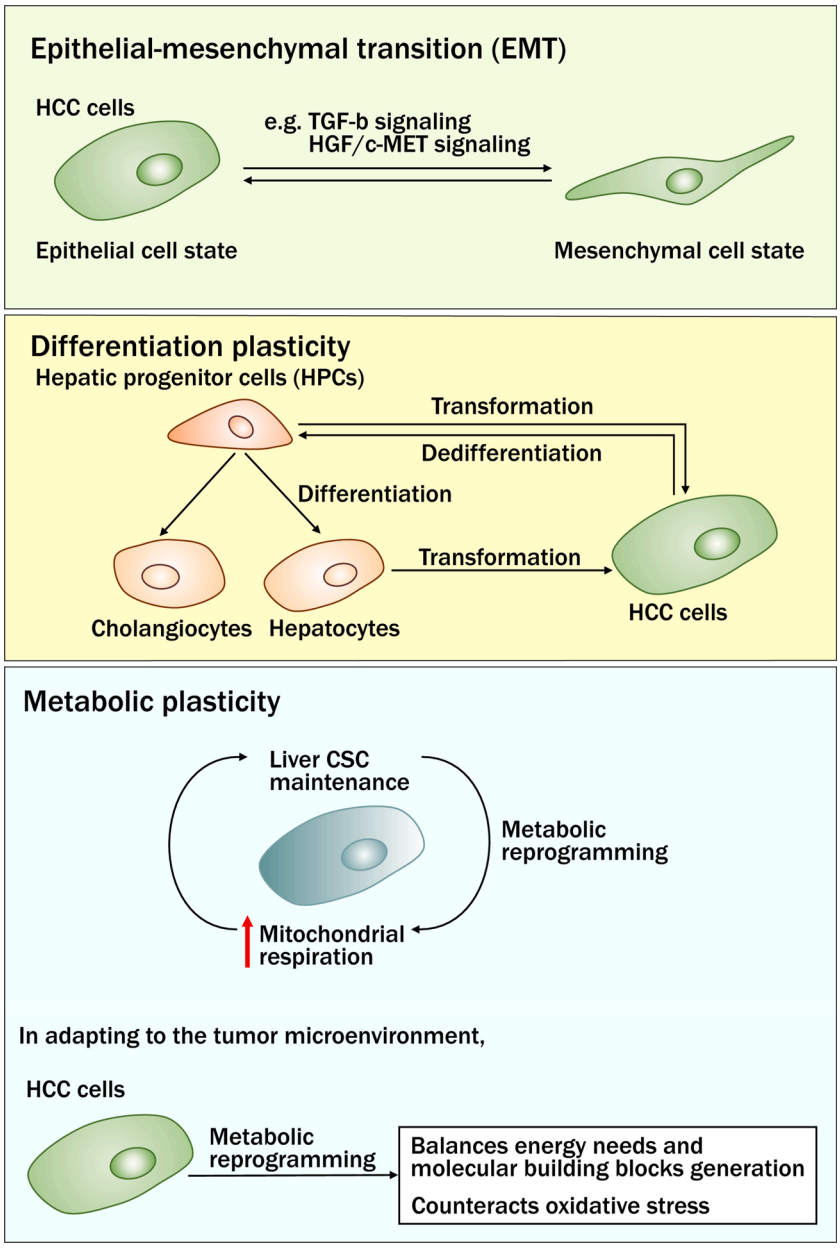

Fig. 3. Cellular plasticity in HCC. Cellular plasticity introduces an additional layer of cellular heterogeneity in HCC. HCC cells acquire other cellular phenotypes by reprogramming themselves directly without dedifferentiating into the pluripotent cell state. A few examples of cellular plasticity HCC cells are illustrated as follows. A) Epithelial-mesenchymal transition. HCC cells acquire mesenchymal phenotype through EMT to facilitate metastasis. B) Differentiation plasticity. HCC cells arise from the transformation of hepatic progenitor cells (HPCs) and hepatocytes. HCC cells also possess the differentiation plasticity to dedifferentiate into hepatic progenitor cells. C) Metabolic plasticity. HCC cells undergo metabolic reprogramming in adapting to the tumor microenvironment. Liver cancer stem cells (CSC) are reprogrammed for increased mitochondrial respiration for their self-maintenance, while the HCC cells metabolically reprogram themselves to balance the energy needs and the generation of molecular building blocks as well as to cope with the oxidative stress to sustain the high proliferative rate and cell survival.

4-dihydrocollidine (DDC) treatment sensitizes Kras–Tp53–mutant hepatocytes to malignant transformation and confers a phenotypic switch specifically favoring an intrahepatic CCA. This highlights the plasticity for trans-differentiation of hepatocytes in bringing about hepatocyte-derived CCA phenotypes. Another study using a mouse model with TP53 and PTEN-knockout in CD133+ HPCs led to the formation of liver tumors with a HCC morphology [78]. However, when the liver cancer organoids derived from the liver tumors were injected into mouse liver, liver tumors with admixed HCC and CCA-like features were generated, suggesting the enriched oncogenic HPCs have great differentiation plasticity to produce heterogeneous liver tumors.

A recent study investigated the plasticity and heterogeneity in biliary epithelial cells (BECs) during hepatic repopulation upon liver injury challenge using sc-RNA-seq [79]. Clustering analysis of the 2344 BECs derived from the normal homeostatic liver and collected by FAC-sorting for positive expression of EpCAM (a BEC marker) identified two subgroups showing differential expression of genes primarily related to YAP signaling. However, upon livery injury induced by DDC, the BECs subpopulations showed enhanced expression of Wnt7a, Wnt7b, Wnt10a, and CD44, but not canonical Wnt/β-catenin marker expression such as LGR5 and Axin2. Furthermore, scRNA-seq on isolated hepatocytes upon DDC-injury revealed a subset of hepatocytes showing YAP-activation and biliary marker expression, indicating possible reprogramming with trans-differentiation of a subset of hepatocytes to ductal cells. It was further shown that YAP abrogation could block such trans-differentiation and subsequent ductular response in hepatocytes upon injury. The observed ability of hepatocyte to transdifferentiate upon liver injury is somehow in line with another study in preserving primary hepatocyte nature in in vitro culture. Sun et al. found that blocking the mechanical tension-induced YAP activity in attached spreading hepatocytes could maintain their differentiated cell state for major hepatocyte functions [80]. On the contrary, without YAP inhibition, the raised mechanical tension by cell spreading promoted dedifferentiation of hepatocytes. These studies highlight the roles of YAP in promoting de- or trans-differentiation ability and thus the differentiation plasticity of liver cells. Whether such plasticity in HCC cells is also mediated by YAP signaling awaits further investigation.

4.3. Metabolic plasticity in HCC

Plasticity in metabolic reprogramming of liver cancer cells has recently been investigated. The more tumorigenic Dt81Hepa1−6 cell line undergoes metabolic adaptation by enhancing glucose uptake, aerobic glycolysis and fatty acid synthesis in the presence of high glucose when compared to its parental counterpart [81]. Also, one study has indicated that SIRT1/MRPS5 is critical in controlling the metabolic switch of the cells from relying on glycolysis to mitochondrial aerobic respiration through enhancement in mitochondria biogenesis [82]. Mitochondrial ribosomal protein S (5MRPS5), a component of complex I of the mitochondrial respiratory chain, is restricted to cytoplasm localization upon deacetylation by SIRT1 to promote mitochondria fusion to promote oxidative phosphorylation (OXPHOS). These indicate that metabolic plasticity can provide liver cancer cells the flexibility to use alternative energy sources to withstand various nutrients or micro-environmental conditions.

A metabolic network analysis was performed by integrating transcriptomic data from The Cancer Genome Atlas (TCGA) on 369 HCC patients and 50 matched non-cancerous samples with the genome-scale metabolic models (GEMs), which are collections of biochemical reactions and associated enzymes and transporters. The study revealed three HCC subtypes with distinct features in survival, gene expression, prognosis and signaling pathways [83]. Interestingly, one subtype is more CC-like than the other two subtypes. The three subtypes may rely on alternative enzymes to catalyze the same reactions, indicating intertumoral heterogeneity in the use of metabolic enzymes in HCC. Whether plasticity is present to allow switches between the use of the alternative enzymes and whether such will bring about changes across the metabolically defined subtypes of HCC are worthy to investigate.

In another study, an integrative multi-omics analysis was performed in HCC for 132 redox metabolism-related protein-coding genes involved in reactive oxygen species (ROS) generation, scavenging and metabolism, metabolism of reducing equivalents, and oxidative stress response [84]. The antagonistic relationship among these genes were found, constituting two clusters of genes, namely the aldehyde dehydrogenase (ALDH2) cluster and the glucose-6-phosphate dehydrogenase (G6PD) cluster, which led to the stratification of the 360 HCCs curated in the TCGA database into two subgroups. As the G6PD gene cluster is also involved in hypoxic response, by validating the results in mouse HCC tumors, it was found that there was increased expression of the G6PD cluster genes including G6PD, ME2, PFKP, GP1, GLS but not for ALDH2 cluster genes such as ALDHB1, ALDH1L1, ALDH2. As HCC usually experiences hypoxia when it grows larger in size, this study suggests the metabolism in HCC is dynamic as the tumor progresses. This highlights the need to investigate the temporal change in metabolic plasticity and heterogeneity in liver cancer cell populations over a period of time under specific tumor microenvironmental changes.

5. Liquid biopsy as an alternative means to assess HCC heterogeneity

Intratumoral heterogeneity imposes a great challenge to extensively detect mutations from tumor tissue and usually results in an underestimation of the number of mutations. Moreover, repeated sampling of tissue biopsies is clinically not feasible. New mutations can arise during the cancer development process and they can also be acquired throughout treatment. Traditional tissue biopsies may have sampling bias in the mutational profile and cause inaccurate evaluation of the disease. Therefore, liquid biopsies may provide an alternative way to mutation discovery that can be applied to early cancer detection, recurrence monitoring, and treatment evaluation (Fig. 4). Liquid biopsy offers a non-invasive means to study the genomic information of the HCC. Currently, circulating tumor DNA (ctDNA), referring to the portion of cell-free DNA (cfDNA) derived from the tumor cells, and CTC are of great research interests and translational potential values in the diagnosis and prognosis of HCC.

5.1. ctDNA in HCC

ctDNA is shed from the necrotic or apoptotic tumor cells and so theoretically, it should contain genetic materials identical to the originating tumor cells. ctDNA has a short half-life between 16 min and 2.5 h [85] and accounts for less than 1% of total cfDNA in the blood [85,86]; preservation and extraction of ctDNA and the ability to discriminate between cfDNA from normal cells and ctDNA from tumor cells are keys to clinical applicability of ctDNA in HCC diagnosis and prognosis. Current advances in ctDNA extraction and NGS platforms have enabled the analyses of ctDNA, while more investigation is needed to identify tumor-specific genetic alteration in ctDNA, including somatic mutations, gene copy number variation, and methylation.

One study found that ctDNA had non-random preferential fragmentation ends as compared to the cfDNA from normal liver cells [87]. Increasing numbers of studies have recently been carried out in ctDNA from HCC patients and details are summarized in Table 1. In brief, TERT promoter mutations [88], CTNNB1 mutations and TP53 R249S mutations [89] have been detected in ctDNA from HCC patients. Aberration of DNA methylation has been implicated in hepatocarcinogenesis and detected in blood samples of HCC patients. These include p15 [90], mSEPT9 [91], APC [92], FHIT [92] and E-cadherin [92] (Table 1). The detection of methylation of ctDNA offers the advantage of early cancer detection [85]. However, most methylation events are non-druggable targets with no specific ways to reverse the specific methylation events in HCC, making the ctDNA methylation mainly for diagnosis and disease monitoring purposes.

5.2. ctDNA & heterogeneity

As compared to cancer biopsy, ctDNA might be a way to reveal

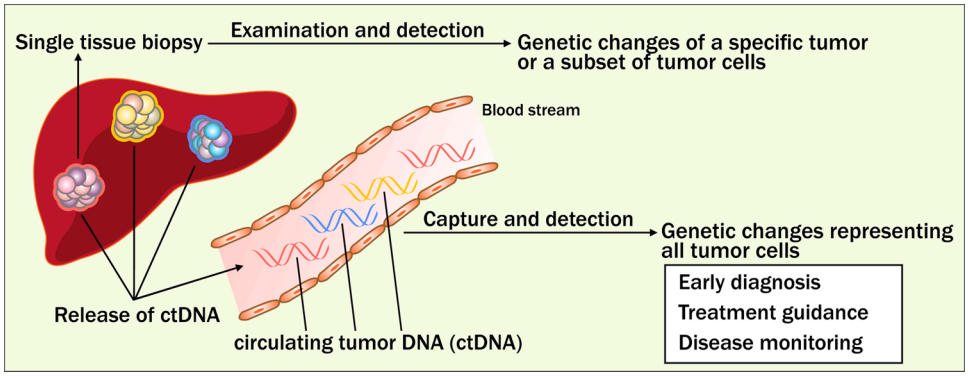

Fig. 4. Liquid biopsy as an alternative means to access HCC heterogeneity.
Single tissue biopsy only allows us to assess the genetic changes of a focal area of a tumor or a subset of tumor cells in human cancer tissues having extensive heterogeneity including HCC. Release of circulating tumor DNA (ctDNA) from the HCC tumors into the bloodstream provide us with a flexible and balanced way to capture and detect the representative genetic changes in the HCC tumors and is highly valuable for early diagnosis, treatment guidance, and disease monitoring.

Table 1
Summary of studies on ctDNA of liver cancers in the recent 5 years.

	Studies	No. of samples analyzed	Study method for the types of genetic alteration	Remarks/ Findings
Study of ctDNA mutations in HCC				
1	Liao et al. (2016) [110]	41	Targeted sequencing for somatic mutations	Tumor-associated mutations in 19.5 % plasma samples
2	Cai et al. (2017) [111]	3 + 1	Targeted sequencing & whole exome sequencing (WES) for somatic mutations	
3	Lim et al. (2018) [112]	27	Targeted sequencing for somatic gene rearrangement/ fusion, mutations and CNV	Mainly focused on RAS mutations in patients receiving refametinib/ refametinib, sorafenib
4	Huang et al. (2016) [88]	48	ddPCR / Sanger sequencing for somatic gene mutations	56.3 % of cases with mutations detected in the ctDNA
5	Ikeda et al. (2018) [113]	14	Targeted exon sequencing and ddPCR for somatic gene rearrangement/ fusion, mutations and CNV	
6	Labgaa et al. (2018) [114]	8	Targeted exon sequencing for somatic gene mutations	15/21 mutations in primary tissues were found in cfDNA
7	Huang et al. (2017) [96]	5	WES & targeted deep sequencing for somatic gene mutations and CNV	Multi-regions of tumors were sampled
8	Ng et al. (2018) [115]	30	Targeted sequencing for somatic gene mutations	65 % patients showed mutation in both plasma ctDNA and the matched HCC tissues
9	Jiao et al. (2018) [116]	218 HCC, 81 cirrhosis	ddPCR & Sanger sequencing for somatic gene mutations	
10	Qu et al. (2019) [117]	Training cohort: 65 HCC & 70 normal; validation cohort: 4 HCC & 327 normal	Targeted sequencing for somatic gene mutations	ctDNA and other markers (e.g. AFP, DCP, HBsAg) were analyzed.
11	Zhen et al. (2019) [118]	3	Targeted sequencing for somatic gene mutations	Monitoring before, during and after TACE: ctDNA mutations unaltered after 1 week TACE but increased sharply after 4 weeks of TACE
12	Howell et al. (2019) [119]	51 HCC, 10 cirrhosis	Targeted ultra-deep sequencing for somatic gene mutations	
13	Ikeda et al. (2018) [120]	26	Targeted sequencing for somatic gene mutations	
14	He et al. (2019) [121]	29	Targeted sequencing for somatic gene mutations	
15	AlunniFabbroni et al. (2019) [122]	13	Targeted sequencing for somatic gene mutations	Prognostic value under study for sorafenib treatment etc; no HBV-positive cases, only one with HCV
16	Li et al. (2020) [123]	50	Targeted sequencing (for discovery) and ddPCR (for validation)	For studying HBV integration
17	Jiang et al. (2018) [87]	1	NGS for tumor-associated ctDNA ends	5.4e6 HCC-associated ctDNA fragment ends coordinates were identified as compared to normal liver donor/ recipient cfDNA ends basing on donor vs recipient-specific SNPs
18	Cai et al. (2019) [124]	1204 HCC, 352 chronic HBV, 958 normal	5- hydroxymethyl cytosine (5hmC)-sequencing for the 5hmC epigenetic modification	5hmC markers in ctDNA enables early HCC detection with superior performance over α-fetoprotein (AFP)
19	Mody et al. (2019) [125]	35	Digital sequencing for somatic gene mutations, SNV, Indel, amplification	A total of 122 unique genetic alterations were observed including the top 10 most common: TP53, TERT, CTNNB1, ARID1A, MYC, BRAF, CCND1, CDK6, MET and EGFR
20	An et al. (2019) [126]	26 HCC, 10 cirrhosis, 10 hepatitis	Targeted sequencing for somatic gene mutations	ROC curve can distinguish HCC from non-HCC (AUC is greater than that of AFP (0.78))
21	Kaseb et al. (2019) [127]	206	NGS for somatic gene mutations, SNV, Indel, amplification	56.9 % patients with mutation in ≥1 actionable gene (MYC, EGFR, ERBB2, BRAF, CCNE1, MET, PIK3CA, ARID1A, CDK6, KRAS)
22	Cai et al. (2019) [128]	34	Targeted sequencing and low-coverage WGS for somatic gene mutations, SNV, CNV	Plasma CNV and SNV levels dynamically correlated with patients' tumor burden in HCC.
Study of ctDNA methylation in HCC				
23	Kisiel et al. (2019) [129]	1089 HCC & 835 normal control	Methylation specific qPCR; target enrichment long-probe quantitative amplified signal (TELQAS) assays	The six-methylated DNA marker (MDM) panel yielded a best-fit AUC of 0.96 with 95 % sensitivity and 92 % specificity.
24				

(continued on next page)

Table 1 (*continued*)

	Studies	No. of samples analyzed	Study method for the types of genetic alteration	Remarks/ Findings
	Xu et al. (2017) [130]	98 HCC, 191 cirrhosis	Targeted bisulfite sequencing	A large clinical cohort for discovering and validating ctDNA methylation markers panel for diagnostic and prognostic prediction.
25	Oussalah et al. (2018) [91]	84	Methylation specific qPCR	
26	Holmila et al. (2017) [131]	Discovery cohort: 29, validation cohort: 33 + 47	Targeted deep sequencing for DNA methylation	
27	Hu et al. (2017) [132]	45	Methylation specific PCR	UBE2Q1 promoter hypomethylation combined with AFP (cut-off of 20 ng/mL) showed sensitivity (58.8 %) and specificity (75.0 %) with ROC 0.720 for discriminating HCC from non-HCC.
28	Mansour et al. (2017) [133]	237 HCC, 257 normal control	Methylation-sensitive restriction enzyme digestion and real-time PCR for DNA methylation	HCC with HCV background; copy number of hypermethylated RASSF1A ctDNA in serum was associated with increased tumor size
29	Wu et al. (2017) [134]	119	Pyrosequencing and qPCR for DNA methylation	A prospective case–control study found TBX2 hypermethylation in ctDNA was associated with increased HCC risk.
30	Wei et al. (2018) [135]	Discovery cohort: 17; validation cohort: 74	Methylation specific PCR and qPCR	Unmethylation of SOCS3 promoter in ctDNA was associated with poorer survival.

heterogeneity in the genetic mutation landscape of cancers. Unlike other cancers such as lung and breast cancers, HCC does not have a well-defined aberrant genetic alteration across patients [93]. The inter-patient heterogeneity in HCC genetics poses challenges to the development of a universal panel of genetic aberration for HCC detection [94]. Furthermore, there is a great variation in the genetic landscape of HCCs with different etiological backgrounds [93]. The inter-patient heterogeneity highlights the importance of personalized precision medicine in the prognosis and management of HCC. To this end, the identification of druggable genetic mutations in the ctDNA is also critical. The continued monitoring of druggable alterations in the ctDNA allows the discovery of any acquired drug resistance over the course of therapy to help determine timely therapeutic interventions. Insight can be drawn from one study that investigated the heterogeneity in ctDNA in relation to acquired drug resistance with tumor biopsy samples in gastrointestinal cancers [95]. In a cohort of 23 patients, actionable genetic alterations were compared, which were validated functionally to account for the resistance mechanisms in previous studies, between the ctDNA and corresponding tumor biopsy DNA collected after cancer progression with acquired drug resistance. They identified more resistance-related genetic alterations in ctDNA than the corresponding tumor biopsy DNA in 18 of 23 (78 %) cases. This indicates tumor lesion biopsy may be insufficient for characterizing the heterogeneity in resistance-related alterations, particularly when patients may harbor multiple subclones of cancer with varying resistance mechanisms. It will be worthy for more investigation as there is a lack of systematic comparison on the intratumoral and inter-patient heterogeneity in genetic alterations between ctDNA and matched tumor tissue DNA in HCC.

Huang et al. investigated the mutation detection efficiency of circulating cfDNA, as compared to a single HCC tumor sample [96]. Whole exome sequencing (WES) and targeted deep sequencing (TDS) were performed in multi-regional tumor samples from 5 patients and their matched preoperative cfDNAs. Tissue specimens were collected from 6 spatially distinct lesion sites of each tumor. Somatic mutations were identified and the proportion of non-ubiquitous variants, i.e. not present in all regions, was defined as intratumoral heterogeneity level. The mutation rate detected by TDS was significantly higher than that detected by WES. TDS of a single tumor specimen could capture ~70 % of the mutational information. Using WES and TDS, 67.4 % and 83.9 % of tumoral variants, respectively, were detected in cfDNA. The detection rate was also higher in HCCs with low intratumoral heterogeneity. However, their detection rates drastically reduced to 17.9 % and 47.2 %, respectively, upon using more stringent variant calling standards (HC variants) in cfDNAs (to mimic the real situation that no matched tumoral mutation profiles are available as reference and more stringent standards are required to control for false positives). The performance of cfDNA was also sub-optimal in detecting potential driver or actionable variants, with 22 % and 26 % of tumoral variants stringently detected by WES and TDS, respectively. Slightly better efficiency could be achieved by cfDNA (84.2 %) than single tumor specimen (78.9 %) in detecting 19 actionable mutations derived from NCI-MATCH trial or indicative of molecularly targeted drugs. HC variants were nearly undetectable in cfDNA five days after hepatectomy. Collectively, these findings suggest that TDS of a single tumor tissue specimen may be an effective strategy to circumvent intratumoral heterogeneity, and cfDNA may serve to complement tumor specimens in unresectable cases and dynamic monitoring of cancer status clinically.

In summary, the intratumoral/ intra-patient heterogeneity of primary tumor can be partially reflected by the ctDNA. However, since the original tumor cells from which the ctDNA is derived are unknown, we cannot study the heterogeneity of the primary tumors at single cell level by merely looking at the ctDNA mutations, unlike most current sc-RNA-seq platforms such as the Chromium 10X platform. Nevertheless, ctDNA can reflect intra- and inter-patient heterogeneity to provide a rationale for personalized precision medicine and drug response monitoring.

5.3. CTC and heterogeneity

CTCs are nucleated tumor cells greater than 4 μm in diameter and positive for EpCAM, cytokeratins 8, 18, and/or 19 and negative for CD45 [85]. They circulate in the bloodstream with a half-life of around 1–2.4 hours and in very low numbers [97] and thus are difficult to be captured. Early-staged cancers may release fewer CTCs [85], making their detection challenging. To detect CTCs in the blood, there are platforms based on enrichment by immuno-affinity and biophysical properties of CTCs and those that do not [98].

Combined CTCs and AFP detection showed improved AUC (0.821) as compared to the CTCs (0.774) and AFP (0.669) alone in a cohort of 113 HCC patients and 57 non-malignant liver disease patients [99]. In addition, studies on HCC patients in different cohorts showed that the presence of CTCs above the defined thresholds in individual studies could predict extrahepatic metastases [100] and recurrence after surgery [101,102]. In another study, the CTCs were stratified into various subtypes according to multiple EMT- or HCC-specific markers, and the clinicopathologic correlation of these different subtypes were studied.

By the CanPatrol system, which is a filtration-based system with RNA probes to label the EMT markers and CD45, it was found mesenchymal CTC (M-CTC) percentage ≥2% and CTC count ≥16 were associated with HCC recurrence and lung metastasis [103]. A similar study on 195 pre-treated HCC patients' blood samples showed that higher counts for M- and hybrid CTC subtypes than the epithelial (E-CTC) subtype significantly correlated with metastasis and advanced tumor stages [104]. This is supported by another study on postoperative blood from a cohort of 62 HCC patients [105], in which M-CTC and hybrid CTC counts were associated with recurrence and M-CTC positivity was an independent risk factor for early recurrence. A study using a microfluidic system coupled to antibody-based capturing of CTCs showed that vimentin-positive CTCs were significantly more frequent in the blood of patients with advanced HCC stage, with AUROC value of 0.89 [106]. These implicate that the M-CTC indicates more aggressive HCC phenotypes.

Besides the heterogeneity in bulk CTCs, the spatial heterogeneity of CTCs in terms of E-, M- or hybrid subtypes distribution has been explored by analyzing the CTCs in blood collected at peripheral vein, peripheral artery, hepatic vein, intrahepatic inferior vena cava and portal vein from 73 HCC patients before tumor resection [70]. Profound spatial heterogeneity of CTCs was found, with CTCs predominantly of epithelial subtype released from the tumor into the hepatic vein and CTCs gradually of mesenchymal subtype along the blood-flow pathway via peripheral artery towards peripheral veins.

However, sole reliance on conventional CTC capturing based on EpCAM immuno-detection can create a bias towards false-negative detection for EpCAM-negative CTCs [107]. In this regard, many studies employed more than one marker, including asialoglycoprotein receptor (ASGPR), epithelial marker (e.g. EpCAM, CK8/18/19) and mesenchymal marker (twist, vimentin) [106]. To study CTC comprehensively, enrichment and separation of CTCs from the background leukocytes, as according to the differential biophysical properties of these cells, have also been developed. These include cell size, mechanical rigidity, density, inertial focusing and dielectric properties of different cells [98]. Immunoaffinity-based positive enrichment of CTCs coupled with negative enrichment to deplete non-CTC leukocytes would further enhance the specificity of CTC detection.

On the other hand, enrichment-free approach involves optical inspection for both biophysical and immunostaining properties of the cells in the blood samples stained with various markers to interrogate each cell for accurate determination and enumeration of CTCs. A study using a labyrinth microfluidic device to capture CTCs by inertial focusing on the counterstained cells for CSC marker CD44, WBCs marker CD45, and HCC markers like glypican-3, glutamine synthetase, HepPar1 [108] found that the positivity rate of CD44 + CTC subtype was associated with advanced HCC stage. Furthermore, through this platform, CTC clusters containing ≥3 cells [70], known as circulating tumor microemboli and deemed more metastatic and resistant to apoptosis than single CTC, were enumerated and found to correlate with advanced tumor stage. Another study utilizing Imaging Flow Cytometry determined the nuclear-cytoplasmic ratio (karyoplasmic ratio) of CD45/DAPI-counterstained cells in 5 mL-peripheral blood on a cohort of 52 HCC patients and found that CD45-negative high karyoplasmic ratio (HKR) cells, which are non-leukocytes with abnormal nuclei and hence defined as CTCs, significantly correlated with the presence of microvascular invasion and poorer recurrence-free survival [109]. More interestingly, such CD45-negative HKR cells can be either EpCAM + or EpCAM- cells, indicating the heterogeneous nature of the CTC populations.

Although CTCs can reflect both intratumoral and inter-patient heterogeneity, the difficulty in capturing CTCs in large quantity reduces the representativeness of CTC heterogeneity for primary tumor heterogeneity. This is further hampered by the lack of common biomarkers to isolate CTCs due to great inter-patient heterogeneity in HCCs. Without suitable CTC-specific biomarkers, one can only base on the absence of CD45 and morphology to distinguish CTCs, and this poses a technical challenge in specifically sorting out CTCs. While ctDNA tends to be released from dying tumor cells, leading to bias in the study of heterogeneity towards dying tumor cells, the fact that portion of CTCs being more prone to apoptosis once shed into the bloodstream might lead to biased capturing of intact CTC with better cellular integrity and cause skewed study in CTC heterogeneity as well.

6. Conclusion

Given the complex architecture of HCC tumor, the investigation and understanding of HCC heterogeneity have been transitioning from the singular understanding restricted to the tumor cells to the diverse interactions across different cell types as a whole within the microenvironment. It is anticipated that the newly generated biological insight would provide a detailed and provocative look at our unmet medical needs in HCC therapy which will eventually inspire better employment of the currently available therapies as well as the development of new treatment paradigms. In the near future, a liquid biopsy system that is quick, reliable, and safe in capturing HCC heterogeneities and reflecting the genetic and cellular signatures of the HCC tumors would become an indispensable part in guiding the choice of treatment and informing disease progression for HCC patient management in the actual clinical setting.

Declaration of Competing Interest

The authors declare that there are no conflicts of interest.

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