Review

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Applications of single-cell multi-omics in liver cancer

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Summary

Primary liver cancer, more specifically hepatocellular carcinoma (HCC), remains a significant global health problem associated with increasing incidence and mortality. Clinical, biological, and molecular heterogeneity are well-known hallmarks of cancer and HCC is considered one of the most heterogeneous tumour types, displaying substantial inter-patient, intertumoural and intratumoural variability. This heterogeneity plays a pivotal role in hepatocarcinogenesis, metastasis, relapse and drug response or resistance. Unimodal single-cell sequencing techniques have already revolutionised our understanding of the different layers of molecular hierarchy in the tumour microenvironment of HCC. By highlighting the cellular heterogeneity and the intricate interactions among cancer, immune and stromal cells before and during treatment, these techniques have contributed to a deeper comprehension of tumour clonality, hematogenous spreading and the mechanisms of action of immune checkpoint inhibitors. However, major questions remain to be elucidated, with the identification of biomarkers predicting response or resistance to immunotherapy-based regimens representing an important unmet clinical need. Although the application of single-cell multi-omics in liver cancer research has been limited thus far, a revolution of individualised care for patients with HCC will only be possible by integrating various unimodal methods into multi-omics methodologies at the single-cell resolution. In this review, we will highlight the different established single-cell sequencing techniques and explore their biological and clinical impact on liver cancer research, while casting a glance at the future role of multi-omics in this dynamic and rapidly evolving field.

Keywords: hepatocellular carcinoma; primary liver cancer; single-cell sequencing; transcriptomics; genomics; epigenomics; proteomics; tumour microenvironment; multi-omics

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Introduction

Primary liver cancer (PLC) remains a significant global health challenge, marked by a persistent increase in both incidence and mortality rates.

PLC comprises a heterogenous group of malignant tumours in the liver, with several histopathological and molecular entities. Hepatocellular carcinoma (HCC), arising from malignant transformation of hepatocytes, accounts for approximately 80-90% of cases.[1]

HCC exhibits high interpatient, intertumoural and intratumoural heterogeneity, characterised by a complex relationship between cancer cells and the surrounding tumour microenvironment (TME). This heterogeneity in cancer, stromal and immune cells plays a pivotal role in tumour development, metastasis, relapse, and drug resistance.[2]

In recent years, single-cell sequencing techniques have emerged as powerful tools in translational liver cancer research for the dissection of the intricate interplay between the TME and cancer cells at an unparalleled degree of resolution.[3] Unimodal approaches to study the genome, epigenome, transcriptome or proteome have already revolutionised our understanding of the different layers in the molecular hierarchy of individual cell types in the multicellular ecosystem of HCC.[3] However, a complete insight into the complex genotype-to-phenotype relationship in patients with HCC will only be possible by integrating various unimodal methods into multi-omics techniques that allow for analysis on the level of the individual cell.[4,5] Therefore, single-cell multi-omics hold promise for advancing our understanding of HCC complexity, and accelerating the comprehension of functional and regulatory mechanisms leading to hepatocarcinogenesis, relapse, metastasis and drug resistance.[6]

In this review, we will provide an overview of the main single-cell sequencing techniques, commencing from the pre-sequencing phase, and traversing the diverse omics layers of molecular hierarchy. Additionally, we will summarise the impact of these technologies on liver cancer research, focusing on their role in elucidating cellular heterogeneity and the immune cell microenvironment. Furthermore, we will highlight their contribution to uncovering the determinants of response or resistance to immunotherapy in liver cancer. Finally, we will cast a glance at the

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future role of multi-omics in this dynamic and rapidly evolving field.

Overview of single-cell sequencing techniques

Every cell in the human body is intricately shaped by a multi-faceted genotype-to-phenotype relationship, where interactions across different hierarchical 'omics' layers dictate cell function. Bulk sequencing approaches have already enabled the analysis of every 'omics' layer of distinct cell populations, providing us with an average genomic, epigenomic, transcriptomic or proteomic profile of a sample.[7] However, averaging signals from large numbers of cells will obscure specific subpopulations or cellular states, which are often involved in disease biology or response to therapy.[8]

In recent years, single-cell sequencing techniques have addressed this challenge by providing insights into the multi-layered status of individual cells, revealing cellular heterogeneity in healthy and pathological states.[9]

Sample preparation and single-cell isolation

The first key steps in single-cell sequencing techniques are the collection and preparation of biological samples, followed by the isolation of individual, viable cells to create a high-quality single-cell suspension (Fig. 1).[10] This pre-sequencing phase determines the molecular integrity of a sample and is therefore a pivotal phase in a successful single-cell sequencing study. Since performing sequencing techniques on fresh tissue can be challenging in clinical research, multiple tissue preservation methods can be applied after sample dissociation to disconnect sampling time from downstream sequencing techniques.

Key points

- Hepatocellular carcinoma (HCC), the most common form of primary liver cancer, exhibits a high level of interpatient, intertumoural and intratumoural heterogeneity, factors that play a pivotal role in hepatocarcinogenesis, relapse and treatment response or resistance.
- Unimodal single-cell sequencing techniques at the genome, epigenome, transcriptome or proteome level have already revolutionised our understanding of the different layers within the molecular hierarchy of individual cells in HCC tumours.
- Single-cell sequencing techniques highlighted the cellular heterogeneity in the tumour microenvironment (TME) of HCC, contributing to a deeper comprehension of tumour clonality, hematogenous spreading, and the important role of the tumour-peritumour junctional zone.
- Single-cell insights have illuminated the intricate interactions of cancer cells with the innate and adaptive immune system, before, during and after treatment. However, major questions remain to be elucidated and biomarkers predicting response to immunotherapy-based regimens are an important unmet clinical need.
- Unravelling the spatiotemporal interactions of the distinct immune components within the TME of HCC is essential in the development of future immunotherapy options and biomarker research.
- Although the application of single-cell multi-omics, that simultaneously integrate various unimodal methods, in liver cancer research has been limited thus far, it holds great promise for the individualised care of patients with HCC.

At present, multiple well-established techniques are used to isolate individual cells before sequencing. FACS (flow-activated cell sorting) is a commonly used, flow-cytometry-based technique where target cells are marked with fluorescent monoclonal antibodies for specific surface markers and sorted

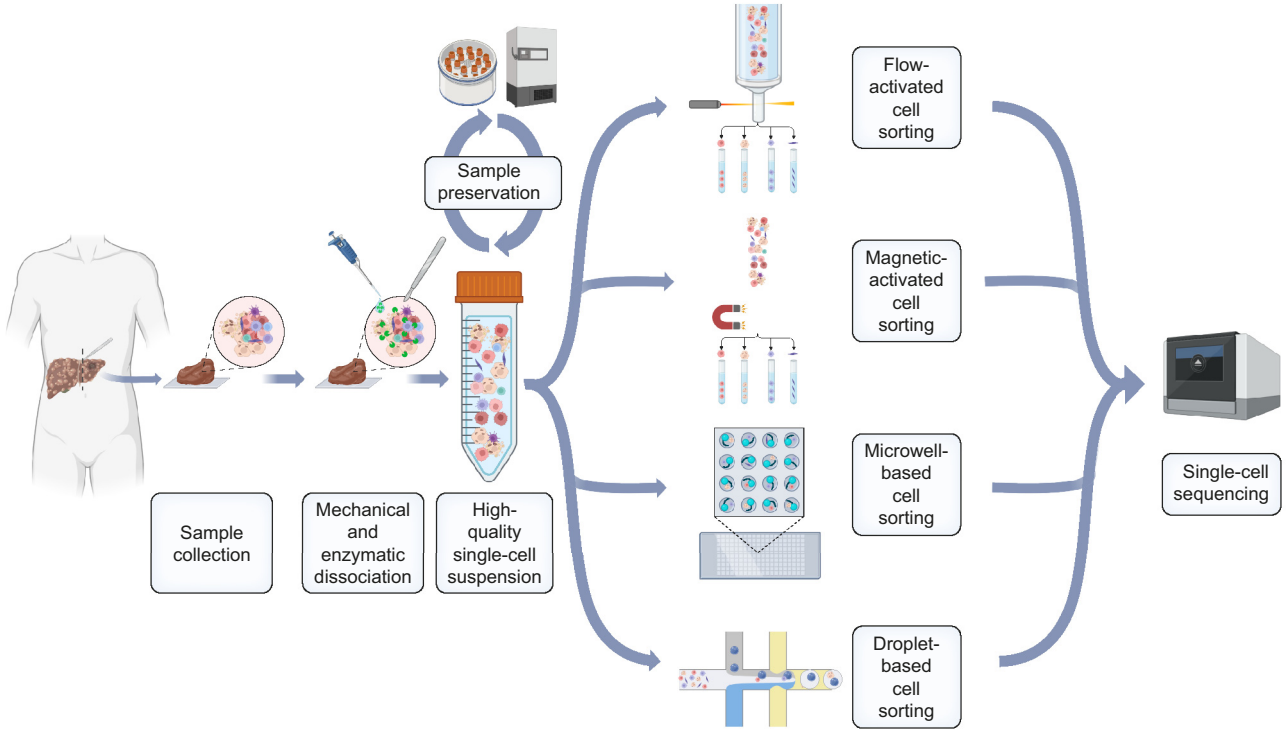

Fig. 1. Pre-sequencing phase of a single-cell sequencing experiment. After sample collection, every step of the pre-sequencing phase determines the molecular integrity of a sample. Single-cell isolation techniques differ in throughput, technical noise, automatization, cost, and complexity.

according to the fluorescent signal. Although this technique allows for the accurate and sensitive sorting of specific cell populations, potential limitations are the requirement for a large starting number of cells, the need for monoclonal antibodies to the specific surface marker and highly trained operators.[11,12] In contrast, MACS (magnetic-activated cell sorting) uses magnetic beads conjugated to antibodies, enzymes, lectins or streptavidins to specifically bind proteins on target cells. An external magnetic field activates the magnetic beads and will result in the separation of the labelled cells. MACS provides a simpler and more cost-effective approach compared to FACS, but it lacks the capability to separate cells based on the expression intensity of a specific molecule.[12]

In recent years, microfluidic technologies have provided new ways of isolating individual cells and can be categorised based on different operating principles.[13] First, in microstructure-based techniques, individual cells are distributed over a high-density array of microwells, ensuring the loading of one individual cell per microstructure.[14] Second, droplet-based techniques produce micrometre-scaled aqueous compartments, encapsulating individual cells together with all the necessary reagents for downstream reactions in an inert carrier oil. These droplets-in-oil-based separation techniques offer significant benefits, including exceptionally high throughput, low technical noise, and an automated workflow that minimises cell stress. Nonetheless, the drawback lies in the requirement for complex instrumentation and the associated high costs.[13,15–17]

After the isolation of individual cells using one of the aforementioned techniques, analysis of the different 'omics' levels can be performed using different sequencing methods (Fig. 2).

Single-cell genome sequencing

Information on genomic aberrations in individual cells or clonally derived cell subpopulations, including copy number variations (CNVs), single-nucleotide variations and structural variants, are essential to better understand human diseases, especially cancer. Following the extraction of DNA from individual cells, multiple rounds of amplification are required to increase the quantity of nucleic acids for downstream analyses.[18] Currently, multiple displacement amplification has emerged as the predominant method for genomic amplification, surpassing PCR, owing to its superior genome coverage and reduced error rates.[19–21]

Given the intricate nature of the human genome, the amplified genomes can be examined at specific loci of interest, across all protein-coding regions known as the exome, or through sequencing the entire genome. This decision is based on the specific research question, with careful consideration of the trade-off between coverage, susceptibility to errors, and total cost.[18]

Single-cell epigenome sequencing

By regulating gene expression without changing the underlying DNA sequences, the epigenome is a crucial determinant of cellular phenotype. The four major epigenetic processes in eukaryotic cells are DNA methylation, histone modification, chromatin accessibility and nucleosome localisation.[22] DNA methylation consists of adding a methyl group at the cytosine residue of a cytosine-guanine (CpG) dinucleotide, leading to the suppression of gene transcription. Bisulphite sequencing, a technique that involves the chemical conversion of unmethylated cytosine into uracil, is a well-established method for DNA methylation assessment at the single-cell level.[23] Methods based on bisulphite sequencing are considered the golden standard due to high conversion efficacy and reproducibility. However, bisulphite treatment causes DNA damage resulting in biased sequencing data.[24] Therefore, bisulphite-independent approaches, using enzymatic DNA methylation conversion, are emerging.[25]

A plethora of post-translational modifications of histones regulate DNA-templated processes.[26] Insights into this second key epigenetic mechanism at the single-cell level can be provided by single-cell chromatin immunoprecipitation sequencing or single-cell DNA adenine methyltransferase identification.[27–29]

The third major epigenetic process is chromatin accessibility and involves the opening of the tightly packed chromatin structures to expose specific DNA sequences for replication and transcription processes.[30] To probe chromatin accessibility in individual cells, scATAC-seq (single-cell assay for transposase-accessible chromatin using sequencing) has emerged as a powerful tool.[31]

Finally, the position of the nucleosome, the structural unit of DNA wrapped around an octamer of histone proteins, is closely associated with the regulation of gene expression.[32] Single-cell micrococcal nuclease sequencing is a high-throughput sequencing technique that uses micrococcal nuclease to degrade nucleosome structures at a single-cell level. When analysing the released DNA, the positioning pattern of nucleosomes can be determined, thereby uncovering the heterogeneity of nucleosome positions between cells.[33]

Single-cell transcriptome sequencing

Single-cell transcriptomics measures the abundance of mRNA in every individual cell, providing deep insights into the heterogeneity and complexity of the transcriptome of different cell phenotypes. Following the isolation and lysis of individual cells, sequencing libraries are created through the conversion of mRNA to complementary DNA (cDNA) and subsequent amplification of the cDNA. Amplification of cDNA can be performed by PCR or using *in vitro* transcription, both successfully implemented in multiple single-cell RNA sequencing (scRNA-seq) methods. Although *in vitro* transcription-based techniques exhibit lower susceptibility to biases, they require additional steps to convert mRNA to libraries making them more time-consuming.[34] Depending on the goal of the experiment, sequencing of the libraries can be done through full-length transcript sequencing or by sequencing the 3′ or 5′ transcript ends. Full-length RNA sequencing covers the entire sequence of RNA molecules and therefore enables identification of splice variants, alternative transcripts, genetic alterations, and genotypes of B- and T-cell receptors.[35–38] To prioritise cost-effectiveness, sequencing can be limited to the 3' or 5' end of the transcript. Opting for 3' sequencing, with its high-throughput nature, proves advantageous for comprehensive gene expression. On the contrary, 5' sequencing provides detailed insights into transcription start sites and isoform diversity, making it particularly valuable for in-depth analyses of alternative splicing events and T-cell receptor clonotypes.[34,39]

In conclusion, a diverse array of scRNA-seq techniques is currently available, varying in sensitivity, accuracy, precision, and cost.[40]

Single-cell proteome sequencing

Since the relationship between mRNA and proteins is not straightforward, single-cell proteomics is useful for clarifying the complex genotype-to-phenotype relationship in eukaryotic

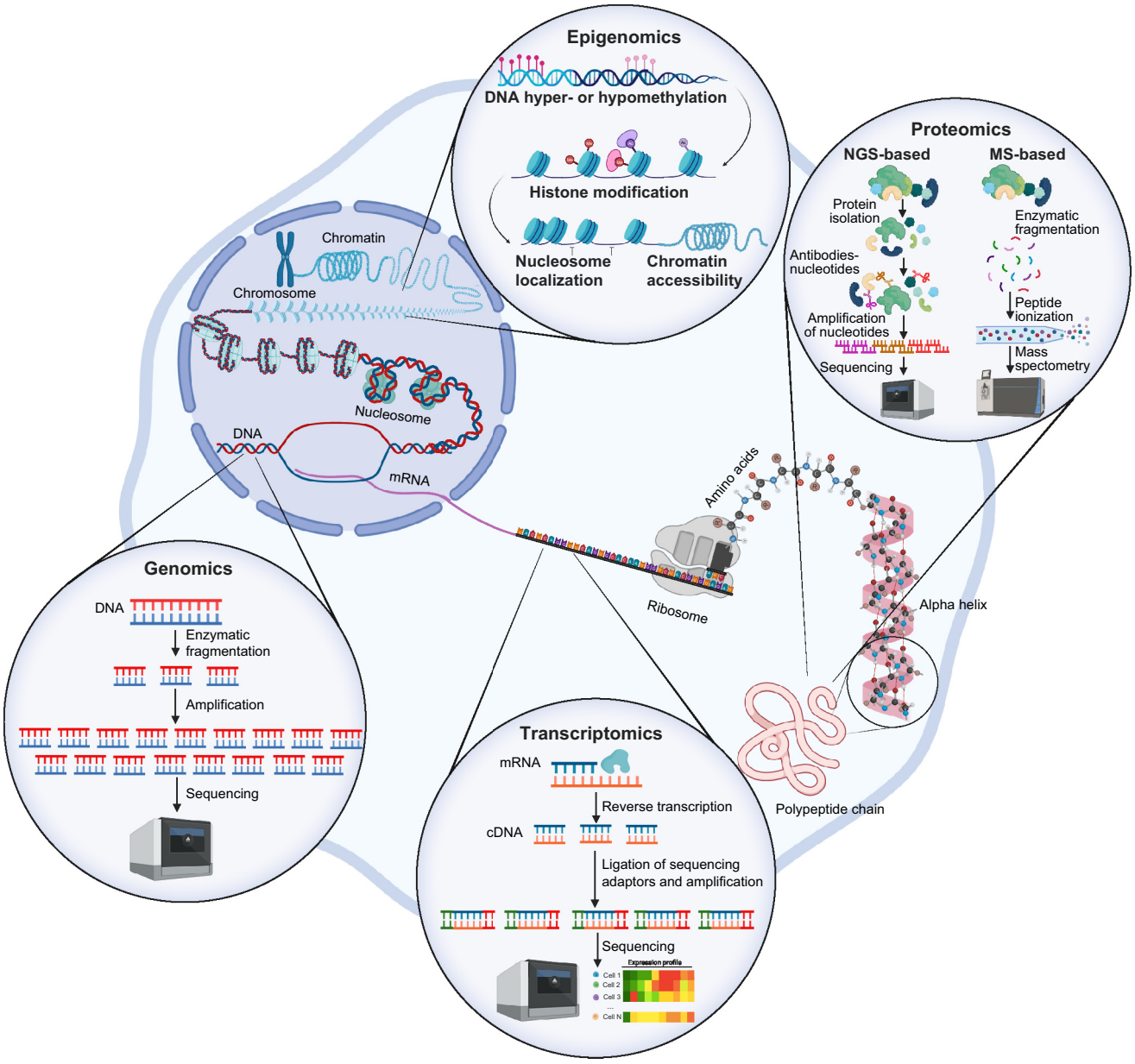

Fig. 2. The different omics layers in a eukaryotic cell. Single-cell sequencing techniques explore each individual omics layer of molecular hierarchy at the resolution of individual cells. On the genotype-to-phenotype journey, going from genomics, to epigenomics and transcriptomics, and finally proteomics, different sequencing techniques can be performed.

cells.[41] Important hurdles in the detection of proteins at the single-cell level arise from the vast range of protein expression within individual cells and the fact that proteins, unlike DNA or RNA, cannot be amplified.[42] Several distinct technologies, subdivided into sequencing- and mass spectrometry-based techniques, have been developed to identify and quantify proteins within individual cells, each with their own unique advantages and barriers.

Next-generation sequencing proteomics is an antibody-based technique which overcomes the difficulties of protein detection via the relatively straightforward detection of antibodies. Nevertheless, this methodology hinges on the accessibility of meticulously characterised antibodies, introducing a potential risk of antibody cross-reactivity.[43]

A second proteomics methodology, independent of specific antibodies, is mass spectrometry-based single-cell proteomics (scMS proteomics), where the protein content of individual cells undergoes enzymatic digestion, producing peptides that are subsequently separated and ionized. Real-time acquisition of mass spectrometry spectra captures profiles of the peptides as they elute sequentially, providing a dynamic and detailed snapshot of the proteomic landscape of individual cells.[44] A notable advantage of scMS proteomics lies in its exploratory nature, as it does not require a predetermined target reagent.[43]

With their intrinsic advantages and hurdles, single-cell next-generation sequencing and scMS proteomics are complimentary approaches for characterising the proteomes of individual cells.

Analysis of a single-cell experiment

Navigating the multitude of computational methods to translate vast single-cell datasets into meaningful biological insights is increasingly challenging. Thus, our aim is to offer a concise overview of the general steps involved in the analysis of a single-cell experiment.

Firstly, the processing phase focuses on generating high-quality cellular data from raw data matrices. This involves a quality control step where entries of low-quality cells are filtered by manually setting thresholds.[45]

Secondly, a normalisation step is essential to correct the raw data in the dataset for variable sampling and sequencing effects by scaling the observable variance to a specified range.[46]

Normalised data may still contain undesired variability stemming from technical and biological covariates, like batch effects, dropout, or cell cycle effects. In the third step, data integration aims to address these confounders.[45]

Fourthly, to alleviate the computational load, diminish background noise, and aid in data visualisation, various techniques are available to reduce the high-dimensionality inherent to these datasets. Typically, the first step involves applying feature selection methods to prioritise biologically relevant features. Subsequently, dimensionality reduction methods further condense the data matrix, aiming to capture the fundamental structure in as few dimensions as feasible.[47] Following this processing phase, modality-specific downstream analyses can be conducted to extract biological insights.

Pitfalls of a single-cell experiment

Single-cell sequencing techniques have become indispensable in liver cancer research, yet realising their full potential requires a nuanced understanding of their inherent challenges.

Owing to the necessity of tissue dissociation into a single-cell suspension, single-cell sequencing techniques come at the cost of losing information concerning physical interactions between cells. Emerging spatial transcriptomic techniques overcome this limitation by mapping the transcriptome of individual cells within intact tissue, thereby unveiling a novel dimension of heterogeneity.

Biological processes are not only spatially heterogeneous but also temporally dynamic. Single-cell sequencing techniques may struggle to capture this temporal aspect, leading to the identification of discrete cell types rather than continuous cell states.[48] To address this issue, innovative computational and technological methods are being developed to map out cell developmental trajectories.[49,50]

Furthermore, variations in expression patterns between cells may indicate transitional states that lie between well-defined cell clusters. Therefore, there is a demand for cell atlases with varying levels of detail, alongside highly adaptable statistical methods for uncovering intricate intermediate cell states.[48]

Single-cell multi-omics

An array of single-cell 'omics' methods has transformed our comprehension of biological processes with unparalleled detail. However, exclusive attention to individual 'omics' layers often neglects the intricate interplay among them. Recent advances in technology and methodology now enable the concurrent assessment of distinct 'omics' layers within individual cells.

As single-cell transcriptomics is the most mature of the single-cell omics methods, it is often linked to other 'omics', to investigate the relationship between gene transcription and every other layer in the molecular hierarchy.[4]

Soon after the introduction of single-cell genomics and transcriptomics, simultaneous profiling of the genome and transcriptome in the same cell was established. So called G&T-seq utilises oligo-dT beads to physically separate mRNA and genomic DNA. Subsequently, both components can be independently amplified and sequenced in parallel.[51] Since genomic DNA and mRNA must be physically separated, this may cause a loss of nucleic acids and therefore an amplification bias. To overcome this hurdle, the recently introduced scONE-seq technique employs distinct barcodes to simultaneously tag mRNA and genomic DNA, enabling concurrent library construction and sequencing. This differential barcoding system facilitates the subsequent differentiation between transcriptomic and genomic information during downstream analysis.[52]

Integrating single-cell transcriptomics with epigenomics provides valuable insights on the query of how identical DNA sequences can yield diverse mRNA expression in distinct cells. ScM&T-seq (single-cell genome-wide methylome and transcriptome sequencing) was one of the first single-cell techniques developed to measure transcriptomic and epigenomic features simultaneously, combining single-cell bisulphite sequencing for DNA methylation and Smart-seq2 for transcription profiles.[53] Combining chromatin accessibility and transcriptional analysis can provide important information on the effect of regulatory elements, such as transcription factor binding sites and enhancer activity on gene expression. scATAC-seq technology is therefore combined with scRNA-seq in plate-based, microfluidic-based or differential indexing technologies.[54–56] Histone modifications exhibit considerable diversity in their cellular specificity and are associated with cell type-specific gene transcription. The role of histone modification on the transcriptome of individual cells can be elucidated by high-throughput techniques, such as the plate-based Paired-Tag and the droplet-based single-cell CUT&Tag.[57,58]

Biological processes, cellular structures and functions revolve around proteins, governed by their foundational molecular layers, and further characterised through post-translational modifications and interactions. In 2017, the introduction of CITE-seq (cellular indexing of transcriptomes and epitopes by sequencing), combined highly multiplexed protein marker detection with transcriptome profiling for thousands of single cells.[59] Proteomics techniques, utilising mass spectrometry, surpass the limitations associated with antigen-specific reagents and enable a more comprehensive and exploratory approach.[60] However, the absence of an available integration method with other 'omics' presents an opportunity for future developments.

Substantial progress has been and will be made on the simultaneous profiling of multiple 'omics' layers of individual cells. Improvements in throughput, an increase in sensitivity and specificity in the characterisation of the individual 'omics' and the incorporation of multiple 'omics' in a single assay are expected soon.[5]

Single-cell omics in hepatocellular carcinoma

The presence of clinical, biological, and molecular heterogeneity is a hallmark of cancer and serves as a crucial factor influencing tumour evolution, treatment response and oncological relapse. Single-cell sequencing technologies have become indispensable to study these aspects in the context of HCC research.

Firstly, HCC is considered one of the most heterogeneous tumour types, displaying substantial interpatient, intertumoural

and intratumoural variability.[61–64] The presence of cellular heterogeneity at the single-cell level within the same tumour location highlights the need to investigate cancer biology at the granularity of the individual cell.[65,66]

Secondly, HCC is not only considered a highly heterogeneous tumour type, but it also arises in a unique immune microenvironment. The liver harbours the largest number of immune cells in the body and maintains a unique immunotolerance for the constant flow of inflammatory stimuli from the gut.[67] Since approximately 90% of HCCs develop on a background of prolonged inflammation, with dysregulation of this tightly controlled immunological network, a closer look at the individual cell types of the hepatic immune system and their communication with malignant hepatocytes is essential to better understand hepatocarcinogenesis and develop new treatment strategies.[1]

Lastly, with the successful introduction of immune checkpoint inhibitors (ICIs) for the treatment of HCC, there is an unmet need to unravel the mechanisms underlying response or resistance to therapy. Single-cell sequencing techniques represent a powerful tool for dissecting these mechanisms, offering insights into cellular and molecular dynamics that influence treatment outcomes and potentially paving the way towards predictive biomarkers.

Cellular heterogeneity in hepatocellular carcinoma

In 2016, an HCC tissue sample was subjected to single-cell sequencing techniques for the first time, laying the foundations for a growing number of studies that have since applied these technologies to HCC samples (Table 1). Hou *et al.* developed a single-cell triple omics sequencing technique to simultaneously analyse the genome, methylome and transcriptome, and applied this technique to 25 single cancer cells from one patient with HCC. Based on CNVs, DNA methylation and RNA expression, two distinct subpopulations of malignant hepatocytes were identified. One subpopulation displayed an increased number of gain-of-function CNVs, expressed more invasive genes, and exhibited a greater tendency to evade immune surveillance. Since this cell subtype accounted for only a minor part of the tumour tissue, it would most likely be concealed in bulk analysis, highlighting the added value of single-cell sequencing to characterise intratumoural heterogeneity and identify cell subpopulations in HCC.[68]

In addition to intratumoural heterogeneity, the transcriptional profiles of malignant hepatocytes exhibited substantial variations when comparing different patients with HCC.[69] Various hypotheses have been proposed to elucidate the heterogeneity observed in malignant hepatocytes, including one that implicates cancer stem cells (CSCs), a specific cell subpopulation bearing stem cell features, as the driving force behind the molecular and biological diversity of HCC tumours. Two studies utilised scRNA-seq to characterise CSC heterogeneity in HCC, both identifying different CSC subpopulations with specific transcriptional signatures.[65,70] Moreover, specific genes within CSC subpopulations were associated with prognosis, indicating that the diversity observed in the transcriptome of HCC CSCs contributes to biological heterogeneity.[65,71]

The clonal evolution model, serving as an alternative hypothesis to elucidate tumoural heterogeneity, posits that individual cells gain a growth advantage through an initial mutation. Subsequent mutations within this original clone may further confer a selective advantage, ultimately giving rise to a novel malignant cell subpopulation.[72] Using single-cell whole-genome sequencing, Duan *et al.* revealed both mono- and polyclonal origins in HBV-related HCC lesions. Tumours with confluent multinodular morphology displayed the highest intratumour heterogeneity and were typically polyclonal. In monoclonal HCC, integration of HBV into the genome of host hepatocytes occurs early during tumour development and can act as an early driver event, ultimately leading to clonal expansion and hepatocarcinogenesis.[73]

Circulating tumour cells (CTCs) have been successfully detected in the bloodstream of patients with HCC, with quantification proving effective in identifying patients with an increased risk of recurrence after curative-intent resection or transplantation.[74,75] The utilisation of scRNA-seq in the identification and characterisation of CTCs in HCC unveiled significant heterogeneity among distinct CTCs and was able to detect known oncogenic drivers.[76] Dissemination of tumour cells in the circulation is a dynamic process, resulting in important temporal heterogeneity. Moreover, Sun *et al.* proved an important spatial heterogeneity in cellular distribution and biological features among CTCs along the circulatory pathway. When entering the circulation at the hepatic vein, CTCs displayed predominantly epithelial characteristics, progressively switching to mesenchymal traits during hematogenous transportation.[77] Due to their non-invasive nature, CTCs hold potential for stratifying patient risk, monitoring disease activity and advancing research on predictive biomarkers.

Malignant hepatocytes are intricately intertwined with the TME, a complex network comprising both cellular and non-cellular components. Beyond the notable heterogeneity in malignant cells, Ma *et al.* demonstrated considerable diversity within the TME, highlighting fluctuating stromal cell compositions across distinct PLC cases. Furthermore, the transcriptomic profiles of non-malignant cells in the TME differed significantly when comparing tumours characterised by high *vs.* low variability in malignant cell populations. Based on transcriptomic data, the authors hypothesised that tumours with a high variability in malignant cells might be more hypoxic, leading to higher expression levels of hypoxia-related genes and contributing to the increased production of VEGFA. The increase in VEGFA could potentially trigger a reprogramming of fibroblasts, endothelial cells, and macrophages, thereby inducing alterations in the TME with potential implications for patient prognosis.[78]

The spatially resolved progressive comparison of TME characteristics from adjacent normal liver tissue across the tumour margin to tumour tissue revealed that a complete fibrous capsule, mainly consisting of fibroblasts and endothelial cells, could lead to higher spatial continuity, lower transcriptome diversity and impaired immune cell infiltration.[79] By further analysing the tumour-peritumour junctional zone, a diversity of intermediate-state cells was identified, including endothelial cells harbouring the molecular characteristics of both tumour-associated and normal endothelial cells.[80]

Single-cell sequencing techniques have proven instrumental in unravelling the intricate interpatient, intertumoural, and intratumoural heterogeneity of HCC, contributing to a deeper comprehension of clonal hepatocarcinogenesis, hematogenous spreading, and the important role of the tumour-peritumour junctional zone.

Tumour immune microenvironment in hepatocellular carcinoma

Cancer represents a complex ecosystem, involving tumour cells surrounded by a multitude of immune cells, cancer-associated

Table 1. Overview of recent single-cell sequencing studies in patients with primary liver cancer.

Study	Sample	Number of patients	Number of human cells analysed	Single-cell technology	Major findings
2016					
Hou *et al.*[68]	HCC tumour tissue	1 HCC	25	scTrio-seq: genomic CNV & DNA methylome & transcriptome	Detection of two subpopulations of cancer cells based on multi-omics.
2017					
Zheng *et al.*[85]	HCC tumour tissue & adjacent normal tissue & peripheral blood	6 HCC	5,063	scRNA-seq & TCR-seq	Identification of 11 functional T-cell subpopulations, with enrichment of specific subsets (*e.g.* exhausted CD8+ T cells & Tregs) in HCC.
2018					
D'Avola *et al.*[76]	HCC CTC	2 HCC	10,234	scRNA-seq	Technology development to identify CTCs in HCC, demonstrating CTC heterogeneity and detection of known oncogenic drivers.
Duan *et al.*[73]	HCC tumour tissue & adjacent normal tissue	3 HCC	111	scWGS-seq	Identification of monoclonality and polyclonality in HBV-related HCC. HBV integration is an early driver event and remains extremely stable during tumour progression.
Sun *et al.*[77]	HCC CTC	73 HCC	NA	scRNA-seq	Epithelial and mesenchymal composition of CTCs differs across different vascular compartments in HCC.
Zheng *et al.*[65]	HCC tumour tissue	1 HCC	3847	scRNA-seq	CSCs in HCC exhibit a high degree of biodiversity and may play a role in tumour heterogeneity and prognosis.
2019					
Ho *et al.*[70]	PDTX model (HCC tumour tissue)	1 HCC	153	scRNA-seq	Study of intratumoural heterogeneity, stemness-related subgroups, and identification of rare cell subpopulations.
Ma *et al.*[78]	PLC tumour tissue	19 PLC (9 HCC & 10 CCA)	5,115	scRNA-seq	HCC and iCCA have a varying degree of transcriptomic diversity. **Higher tumour transcriptomic diversity** is associated with worse patient outcomes.
Zhang *et al.*[97]	HCC/iCCA tumour tissue & adjacent normal tissue & lymph node & peripheral blood & ascites	15 HCC + 1 iCCA	77,321	scRNA-seq	High-resolution dynamic immune landscape in HCC. LAMP3+ DCs can migrate from tumour to hepatic lymph nodes. Macrophage subsets in tumours show distinct states and can egress to ascites.
Zhang *et al.*[103]	HCC tumour tissue & adjacent normal tissue & peripheral blood	8 HCC	NA	scRNA-seq & single-cell mass cytometry	Bulk multi-omic analysis with a comparison with single-cell transcriptomics. Proposition of a novel immunophenotypic classification of HCC into three subtypes: immunocompetent, immunodeficient and immunosuppressive.
2020					
Li *et al.*[87]	HCC tumour tissue & adjacent normal tissue & peripheral blood	15 HCC	150,000	scRNA-seq & single-cell mass cytometry	Identification and trajectory analysis of $CD8^+PD\text{-}1^+CD161^+$ and $CD8^+PD\text{-}1^+CD161^-$ T cells in HCC.
Liu *et al.*[112]	HCC tumour tissue & adjacent normal tissue	13 HCC	8,047	scRNA-seq	Presence of an important heterogeneity in $CD4^+$ and $CD8^+$ T-cell exhaustion and their correlation with clinical outcome. Infiltration of **$CD8^+$ T- or $CD8^+$Tex cells** was correlated to overall or recurrence-free survival following resection.
Losic *et al.*[66]	HCC tumour tissue & adjacent normal tissue	2 HCC	38,553	scRNA-seq	Bulk multi-omics intratumoural heterogeneity analysis with RNA-seq, DNA-seq, TCR-seq and SNP array data. Detection of different clonal expansion of the adaptive immune response in distinct regions of the same tumour. Single-cell RNA-seq identifies strong regional transcriptomic differences.
Massalha *et al.*[113]	Tumour tissue & adjacent normal tissue	3 CRC metastases & 2 CCA & 1 benign cyst	7,947	scRNA-seq	Characterisation of cell types in malignant and non-malignant liver lesions.
Sharma *et al.*[100]	Human foetal liver & HCC tumour tissue & adjacent normal tissue	4 foetuses & 14 HCC	133,600	scRNA-seq	Single-cell atlas of human liver from development to HCC. Identification of an **onco-foetal ecosystem** in HCC with an important role for **VEGF/NOTCH signalling**, resulting in the presence of immunosuppressive $FOLR2^+$ TAMs.

(*continued on next page*)

Table 1 (*continued*)

Study	Sample	Number of patients	Number of human cells analysed	Single-cell technology	Major findings
Song *et al.*[114]	HCC tumour tissue & adjacent normal tissue	7 HCC	41,698	scRNA-seq	Characterisation of immune microenvironment of HBV/HCV-related HCC and identification of novel macrophage and T-cell subpopulations. **CD8$^+$ T cells with high secretion of XCL1** were correlated with better prognosis.
Zheng *et al.*[89]	HCC tumour tissue & tumour margin & adjacent normal tissue	13 HCC	17,816 (scRNA-seq) 17,432,600 (scCyTOF)	scRNA-seq & TCR-seq & single-cell mass cytometry	CD4$^+$/CD8$^+$T cells are enriched in the tumour margins with synergetic expression of PD-1/HLA-DR/ICOS/CD45RO. Further characterisation of 11 different CD4$^+$/CD8$^+$T-cell subpopulations.
2021					
Dong *et al.*[69]	HCC tumour tissue & adjacent normal tissue	6 HCC	405	scRNA-seq	Identification of heterogeneous subclones in HCC tissue. **MLX-interacting protein like (MLXIPL)** was commonly upregulated and associated with poor outcome.
Ho *et al.*[93]	HCC tumour tissue	8 HCC	43,645	scRNA-seq	Characterisation of tumour heterogeneity and immune microenvironment in HBV-associated HCC. TAMs were found to suppress T-cell infiltration and regulate the immunosuppressive environment through **TIGIT-NECTIN2** interactions.
Ma *et al.*[115]	PLC tumour tissue	37 PLC (25 HCC & 12 CCA)	56,721	scRNA-seq	**Tumour cell state heterogeneity** is associated with patient prognosis in PLC. SPP1 expression was correlated with tumour cell evolution and microenvironmental reprogramming.
Sun *et al.*[106]	HCC tumour tissue & adjacent normal tissue	18 HCC	16,498	scRNA-seq	Early relapsed HCC have a distinct immune ecosystem with reduced Tregs, increased DCs, and increased intratumoural infiltration of CD8$^+$ T cells. CD8$^+$ T cells in relapsed HCC have an innate-like, low cytotoxic and low clonal expansion phenotype.
Sun *et al.*[116]	HCC CTC	10 HCC	131	scRNA-seq	Transcription profiles of CTCs were associated with stress response, cell cycle and immune evasion signalling. The chemokine **CCL5** was identified as an important mediator of CTC immune evasion.
Vong *et al.*[117]	HCC tumour tissue & adjacent normal tissue & peripheral blood	4 HCC	17,176	scRNA-seq	Cell type-specific gene signature score for hepatocyte-like cells declined significantly after tumour resection. **The cell type-specific gene signature score** for hepatocyte-like cells and cholangiocytes trended with survival.
Wu *et al.*[79]	PLC tumour tissue & tumour margin & adjacent normal tissue & peripheral blood	7 PLC (5 HCC & 1 CCA & 1 cHCC-CC)	NA	Spatial transcriptomics	Characterisation of spatial transcriptomic heterogeneity in PLC. The tumour capsule potentially affects intratumour spatial cluster continuity, transcriptome diversity, and immune cell infiltration.
2022					
Guo *et al.*[118]	HCC tumour tissue & adjacent normal tissue	14 HCC	28,975	scDNA-seq & ploidy-resolved scDNA-seq & scRNA-seq	The accumulation of copy number alterations follows a dual-phase copy number evolution model with a punctuated phase and a gradual phase. HCC with a longer gradual phase were more severe.
Hao *et al.*[94]	HCC tumour tissue & adjacent normal tissue & peripheral blood	4 HCC	212,494	scRNA-seq	APOC1 was overexpressed in TAMs of HCC tissue. The expression of **APOC1** was found to be negatively correlated with the expression of PD-1/PD-L1. Inhibition of APOC1 can promote the transformation of M2 macrophages into M1 macrophages via the **ferroptosis pathway**.
Liu *et al.*[119]	HCC tumour tissue & adjacent normal tissue & peripheral blood	4 HCC	120,497	scRNA-seq	Only 3/5 subsets of NK cells identified in healthy liver tissue were present in HCC. The cytotoxic NK cell subsets were absent in HCC tissue.

(*continued on next page*)

Table 1 (*continued*)

Study	Sample	Number of patients	Number of human cells analysed	Single-cell technology	Major findings
Lu *et al.*[95]	HCC tumour tissue & adjacent normal tissue & portal vein tumour thrombus & metastatic lymph node	10 HCC	71,915	scRNA-seq	Characterisation of the multicellular ecosystem of HCC from four different tissue sites. There is an enrichment of central memory T cells in early tertiary lymphoid structures. $MMP9^+$ macrophages are terminally differentiated TAMs and $PPAR\gamma$ is the pivotal transcription factor driving their differentiation. Identification of seven different microenvironment-based HCC subtypes, with different prognosis.
Ma *et al.*[120]	PLC tumour tissue & tumour margin & adjacent normal tissue	7 PLC (4 HCC & 3 CCA)	112,506	scRNA-seq	Identification of a continuous communication between malignant cells and tumour-associated immune cells. The link between tumour cells and macrophages via ligand–receptor interactions from **LGALS9-SLC1A5 and/or SPP1-PTGER4 signalling** pairs appears to be a stable molecular feature to define intratumoural heterogeneity.
Xue *et al.*[2]	PLC tumour tissue	124 PLC (79 HCC & 25 CCA & 7 cHCC-CC & 13 others)	1,092,172	scRNA-seq	Identification of five different tumour immune microenvironments: immune activation, immune suppression mediated by myeloid or stromal cells, immune exclusion, and immune residence phenotypes. TAN populations in the myeloid cell enriched subtype are associated with an unfavourable prognosis. **$CCL4^+$, $SPP1^+$ and $PD\text{-}L1^+$ TANs** have a pro-tumour phenotype.
2023					
Cappuyns *et al.*[109]	HCC tumour tissue & peripheral blood	44 HCC	366,754	scRNA-seq & TCR-seq	Characterisation of the intratumoural and peripheral immune context of patients with aHCC treated with atezolizumab-bevacizumab. Tumours from patients with durable response are enriched for **$PD\text{-}L1^+CXCL10^+$ macrophages** that are predicted to attract peripheral **$CXCR3^+CD8^+$ TEM cells**. $CD8^+$ TEM preferentially differentiate into clonally expanded **$PD\text{-}1^-CD45RA^+CD8^+$ TEM** cells with pronounced cytotoxicity. In responders, $CD8^+$ TEMRA cells display a **high degree of T-cell receptor sharing** with blood.
Chen *et al.*[121]	HCC tumour tissue & adjacent normal tissue	20 HCC	317,558	scRNA-seq & TCR-seq	True and *de novo* HBV-related HCC recurrence have a distinct tumour immune microenvironment. Tumour-specific $CD8^+$ T cells displayed cytotoxic and exhausted phenotypes in *de novo* recurrences, while those in truly recurrent lesions showed a memory phenotype with weak cytotoxicity.
Chen *et al.*[122]	HCC tumour tissue & adjacent normal tissue	12 HCC	102,735	scRNA-seq	Molecular characterisation of scirrhous hepatocellular carcinoma revealed a hypoxia-driven tumour-stroma remodelling and an immunosuppressive tumour microenvironment.
Craig *et al.*[123]	PLC tumour tissue	16 PLC (13 HCC & 3 CCA)	18,631	scATAC-seq	Transcription factor motif enrichment levels of 31 transcription factors strongly discriminate HCC from CCA. The POU motif family is associated with poor prognosis in CCA.
Kan *et al.*[110]	HCC tumour tissue	6 HCC	22,292	scRNA-seq	High expression of the transcription factor **MZF1** is correlated with an immunosuppressive microenvironment. MZF1 promotes PD-L1 ubiquitination via CDK4.
Ke *et al.*[124]	HCC tumour tissue & adjacent normal tissue	5 HCC	46,789	scRNA-seq	Single-cell atlas of microvascular invasion in HCC. Identification of cycling T cells, $LAMP3^+$ DCs, $TREM2^+$ macrophages, myofibroblasts, and arterial 1 endothelial cells as critical cell types for the immunosuppressive and pro-metastatic microenvironment.

(*continued on next page*)

Table 1 (*continued*)

Study	Sample	Number of patients	Number of human cells analysed	Single-cell technology	Major findings
Magen *et al.*[107]	HCC tumour tissue & adjacent normal tissue	29 HCC	918,811	scRNA-seq & spatial transcriptomics	Response to ICI was correlated with the clonal expansion of intratumoural **CXCL13$^+$CH25H$^+$IL-21$^+$PD-1$^+$CD4$^+$ T helper cells** and **Granzyme K$^+$ PD-1$^+$ effector-like CD8$^+$ T cells**. Progenitor CD8$^+$ T cells interact with CXCL13$^+$ T helper cells around dendritic cells enriched in maturation and regulatory molecules. These cellular triads control the differentiation of tumour-specific progenitor exhausted CD8$^+$ T cells following ICI.
Meng *et al.*[111]	HCC tumour tissue	7 HCC	31,672	scRNA-seq	**CD10$^+$ALPL$^+$ neutrophils** exhibit immunosuppressive features in the TME and contribute to tumour resistance to anti-PD-1 treatment in patients with HCC. Irreversible T-cell exhaustion in anti-PD-1-resistant patients is triggered by CD10$^+$ALPL$^+$ neutrophils.
Ruf *et al.*[90]	HCC tumour tissue & adjacent normal tissue	8 HCC	44,454	scRNA-seq	MAIT cells in HCC show impaired tumour infiltration, dysfunction, and loss of cytotoxicity. MAIT cell function is impacted by TAMs trough interaction at the tumour margin.
Tang *et al.*[125]	HCC tumour tissue & adjacent normal tissue	7 HCC	32,506	scRNA-seq	**FABP1** is overexpressed in TAMs of stage III HCC compared with stage II HCC. FABP1 interacted with PPARG/CD36 in TAMs to increase fatty acid oxidation, creating a FABP1-dependent immunosuppressive environment in HCC.
Zhang *et al.*[126]	HCC tumour tissue	7 HCC	NA	Spatial transcriptomics	Characterisation of the TME of patients with HCC receiving neo-adjuvant cabozantinib and nivolumab. Responding tumours were enriched for **immune cells** and **cancer-associated fibroblasts** with pro-inflammatory signalling relative to the non-responders.
Zhou *et al.*[71]	HCC tumour tissue	5 HCC	36,085	scRNA-seq	NRCAM is highly expressed in liver CSCs with MYC activation in metastatic HCC. NRCAM facilitates migration and invasion of liver CSCs, enhancing the ability to metastasise.
Zhou *et al.*[80]	PLC tumour tissue & tumour margin & peripheral blood & lymph node	7 PLC (2 HCC & 3 CCA & 2 cHCC-CC)	289,156	scRNA-seq & spatial transcriptomics	Characterisation of single-cell and spatial transcriptomic architecture of PLC. CCA and HCC exhibit distinct tumour-specific features. There is a diversity of intermediate-state cells in the tumour margin, including intermediate-state endothelial cells with molecular characteristics of both tumour-associated and normal endothelial cells.
Zhou *et al.*[127]	HCC tumour tissue & adjacent normal tissue	5 HCC	104,800	scRNA-seq	**TNF signalling**, derived from MAIT cells, promoted immunosuppression by enhancing the expression of **TNFRSF1B on Tregs** in patients with HCC resistant to lenvatinib plus anti-PD-1 antibodies.
2024					
Sun *et al.*[128]	Metastatic HCC tumour tissue	5 HCC	23,713	scRNA-seq & spatial transcriptomics	Metastases without Wnt mutations are enriched with immunosuppressive B cells that mediate terminal exhaustion of CD8$^+$ T cells through the **HLA-E:CD94-NKG2A checkpoint axis**.

The presented overview of studies is derived from a comprehensive search on PubMed and through references of relevant articles. Only studies providing new single-cell datasets in PLC were included in this overview.
Most important pathways or potential biomarkers are marked in bold.
aHCC, advanced hepatocellular carcinoma; CCA, cholangiocarcinoma; cHCC-CC, combined hepatocellular carcinoma-cholangiocarcinoma; CNV, copy number variations; CRC, colorectal carcinoma; CSCs, cancer stem cells; CTCs, circulating tumour cells; DC, dendritic cell; FABP1, fatty acid binding protein 1; HCC, hepatocellular carcinoma; MAIT, mucosal-associated invariant T cells; MZF1, myeloid zinc finger 1; NK, natural killer; NRCAM, neuronal cell adhesion molecule; PDTX, patient-derived tumour xenograft; PLC, primary liver cancer; scWGS, single-cell whole-genome sequencing; SNP, single-nucleotide polymorphism; TAMs, tumour-associated macrophages; TANs, tumour-associated neutrophils; TCR, T-cell receptor; TEM, effector-memory T; TEMRA, CD45RA+ effector-memory T cells; Tregs, regulatory T cells.

fibroblasts (CAFs), endothelial cells, pericytes, and various tissue-resident cell types, all embedded in an altered, vascularised extracellular matrix.[81]

The immune component within the TME of HCC is characterised by substantial heterogeneity, featuring diverse cell populations from both the innate and adaptive immune system, intricately balancing immunosuppressive and immune-enhancing signals.[82]

Due to their strong cytotoxic effector function, $CD8^+$ T cells are generally considered one of the most important antitumoural components of the TME. However, intratumoural $CD8^+$ T cells exist in different cell states, often with an exhausted or dysfunctional phenotype.[83] $CD4^+$ T cells are also required for an efficacious antitumoural immune response; however, they play a dual role in cancer. $CD4^+$ helper T cells exhibit their anti-tumourigenic functions directly (by eliminating tumour cells via cytolytic mechanisms) and indirectly (by helping cytotoxic $CD8^+$ T cells and B cells). Conversely, $CD4^+$ helper T cells can also adopt a pro-tumourigenic stance by secreting anti-inflammatory mediators. Additionally, the subset of $CD4^+$ regulatory T cells (Tregs) was identified as a highly immunosuppressive faction, effectively curtailing anti-tumour immunity through diverse mechanisms.[81,84]

Using scRNA-seq, Zheng *et al.* characterised the landscape of infiltrating T cells in HCC. They unveiled 11 functionally distinct subpopulations and delineated developmental trajectories ranging from effector populations to intermediate states ultimately progressing to exhausted $CD4^+$ or $CD8^+$ T cells.[85] This comprehensive characterisation of the landscape of infiltrating T cells in HCC not only provided valuable insights into the immune microenvironment but also emphasised that major questions regarding the working mechanisms of immunotherapy remain. Immunotherapy focusses on the reactivation of a suppressed or dormant anti-tumour response in the TME, as cancer cell signals lead to T-cell exhaustion, characterised by a loss of effector functions and an increased expression of diverse inhibitory receptors. Notably, PD-1, an inhibitory receptor on activated T cells, stands as an important target for ICIs, aiming to reverse this dysfunctional state and reinvigorate pre-existing immune responses. Owing to the importance of ICIs blocking PD-1, the focus of single-cell studies in HCC shifted to a deep phenotypic characterisation of $CD8^+$PD-1^+ T cells.[86] Based on scRNA-seq and mass spectrometry, $CD8^+$ T cells were further classified into subclusters exhibiting heterogenic expression of PD-1 and CD161. Notably, the cytotoxic activity of T cells, characterised by elevated expression levels of TNF-α, IL-2, and PRF, was higher in CD161-expressing $CD8^+$PD-1^+ T cells than in CD161-negative $CD8^+$PD-1^+ T cells. This finding underscores that PD-1 expression alone is insufficient to determine the functional status of $CD8^+$ T cells in HCC. The observation of a higher proportion of cytotoxic $CD8^+$PD-$1^+$$CD161^+$ T cells in non-tumour adjacent liver tissue, in contrast to a higher proportion of more exhausted $CD8^+$PD-$1^+$$CD161^-$ cells in tumour tissue, suggests an intricate spatial transition process within the immune microenvironment.[87] This transition seems to navigate from an activated state to a more exhausted condition, highlighting the nuanced and complex dynamics at play in the immune response within the tumour and its adjacent environment.

Spatial immune heterogeneity and the importance of the tumour-peritumour junctional zone was confirmed by the identification of important differences in immune cell composition between non-tumour liver tissue, tumour margin and HCC tumour tissue by single-cell mass cytometry. The role of the tumour margin as an immunological transition zone was highlighted by the identification of a unique T-cell composition with a small subpopulation of $CD4^+CD8^+$ T cells. $CD4^+CD8^+$ T cells are a well-described T-cell developmental stage within the thymus; however, such cells are increasingly being identified in numerous disease settings.[88] The function and origin of $CD4^+CD8^+$ T cells remain controversial, although single-cell findings suggest that, in HCC, they originate from infiltrating $CD4^+$ or $CD8^+$ single-positive T cells and most subclusters have an active antitumoural phenotype.[89]

Mucosal-associated invariant T (MAIT) cells, another specific subtype of T cells, were also found to accumulate at the tumour margin.[90] MAIT cells are innate-like T cells that recognise non-peptide antigens presented by MR1, a monomorphic major histocompatibility complex class I–like protein.[91] Whether MAIT cells promote or suppress cancer remains elusive, but an increased number of HCC-infiltrating MAIT cells was found to be correlated with poor clinical outcome, most likely due to a functional tumour-promoting reprogramming.[92] Other than their defective capacity to infiltrate the liver tumour, leading to an accumulation at the tumour margin, they showed a progressive loss of cytotoxicity within the tumour. Through a combination of *ex vivo* experiments and spatial single-cell imaging techniques, it was demonstrated that MAIT cell cytotoxic function was impaired by $CD163^+$PD-$L1^+$ tumour-associated macrophages (TAMs) through a PD-L1-dependent mechanism. These insights suggest that MAIT cells play a role in the mechanisms of ICIs targeting PD-1/PD-L1, offering potential new avenues for therapeutic intervention.[90]

TAMs not only impair the functionality of MAIT cells, but they also represent a highly diverse myeloid immune cell population with both pro- and anti-tumourigenic functions.[81] Multiple single-cell studies have provided insights into the functions of TAMs in HCC. First, the presence of TAMs in the TME, particularly the cancer-promoting, anti-inflammatory M2 TAMs, was inversely related with the presence of tumour-invading lymphocytes, suggesting that TAMs hinder T-cell infiltration.[93] Second, potential mechanisms of pro-tumourigenic differentiation of TAMs were identified in HCC. Hao *et al.* demonstrated that APOC1 was overexpressed in TAMs of human HCC tissues compared to adjacent liver tissue. *In vitro* and mouse experiments showed that inhibition of APOC1 reversed the pro-tumourigenic M2 phenotype to the pro-inflammatory M1 phenotype via the ferroptosis pathway, inducing a more anti-tumourigenic TME.[94] Additionally, Lu *et al.* characterised the heterogeneity of TAMs in the TME of HCC and identified $MMP9^+$ TAMs as terminally differentiated from different subpopulations. *In vitro* experiments showed that PPARγ had an important function as a driver of the differentiation of TAMs towards terminally differentiated $MMP9^+$ TAMs and subsequently induced HCC cell migration, invasion, and tumour angiogenesis.[95]

The myeloid compartment in the TME of HCC comprises more than only TAMs. Dendritic cells (DCs), a diverse group of antigen-presenting cells, play critical roles in initiating and regulating both innate and adaptive immune responses. By conveying information from the TME to other immune cells, particularly T cells, DCs possess the potential to mould anti-tumour immunity.[96] Heterogeneous subsets of DCs were identified in the TME of HCC and a subcluster of $LAMP3^+$ DCs appeared to be the mature form of conventional DCs. This specific subset of DCs expressed the highest number of ligands that could interact with T or natural killer cells and could easily migrate from tumour sites to lymph nodes, indicating their important role in priming and activating T cells in an antitumoural immune response.

However, analysis of the cancer genome atlas indicated a strong correlation between the signature of LAMP3$^+$ DCs and exhausted T cells or Tregs, hypothesising a role of LAMP3$^+$ DCs in tumour-induced immune evasion.[97]

Beyond immune cell types, endothelial cells and fibroblasts, respectively lining the blood vessels and remodelling the extracellular matrix, also have the potential to modulate the immune microenvironment in cancer.[98,99] Remarkably, through single-cell transcriptomics and spatial transcriptomics of human foetal liver, HCC tissue and adjacent liver tissue, Sharma *et al.* identified several features of the TME of HCC reminiscent of foetal liver development. They hypothesised that activation of the VEGF and NOTCH signalling pathways would result in embryonic-like PLVAP$^+$ endothelial cells and FOLR2$^+$ TAMs, respectively, maintaining the immunosuppressive TME of HCC.[100] More recently, re-analysis of these data identified a population of onco-foetal POSTN$^+$ CAFs, exhibiting spatial proximity and crosstalk with previously described onco-foetal cell types, influencing early relapse and response to immunotherapy.[101] Furthermore, a recent single-cell sequencing study conducted on both mouse and human liver tissue highlighted the role of CAFs in hepatocarcinogenesis. This study revealed a dynamic shift in subpopulations of hepatic stellate cells, the primary source of liver fibroblasts, during chronic liver disease, which was linked to a transition towards a tumour-promoting TME.[102]

Due to the substantial heterogeneity within the immune component of the TME in HCC and the diverse responses to immunotherapy, efforts are being made to categorise HCC in different immune subtypes that can be linked to treatment outcome. A first immunophenotypic classification was suggested by Zhang *et al.* in 2019. Employing a 'multi-omic' clustering approach for immune cells in the TME of HCC, they identified three distinctive subtypes. First, immunocompetent tumours exhibited normal T-cell infiltration levels and balanced cytokine and chemokine expression. Second, immunodeficient tumours displayed reduced lymphocyte infiltration but higher levels of DCs, limiting the expected impact of ICI. Third, immunosuppressive tumours showed elevated frequencies of Tregs, cancer-promoting M2 TAMs and high expression levels of immunosuppressive molecules, suggesting a potent effect of ICIs in restoring the antitumoural immune response.[103] In 2022, Xue *et al.* proposed an alternative classification with five scRNA-seq-based immune microenvironment subtypes. A key distinction was the subdivision of immunosuppressive tumours, based on whether myeloid or stromal cells predominantly contributed to a pro-tumoural phenotype. The fifth category, associated with favourable outcome, comprised tumours with predominantly liver-resident clusters, including Kupffer cells, liver sinusoidal endothelial cells and resident natural killer cells.[2]

Though immunophenotypic classifications are not yet utilised in clinical practice, single-cell sequencing-derived insights provide the foundations for the development of future immunotherapy options and biomarker research.

Treatment response in hepatocellular carcinoma

With the introduction of tyrosine kinase inhibitors and ICIs, the treatment landscape of HCC has changed dramatically during the last 15 years. Despite great advances in the characterisation of the TME of HCC, factors associated with treatment response or resistance remain to be elucidated.

Patients with early HCC are eligible for resection. Nevertheless, most of these patients experience recurrence, pragmatically classified into early or late recurrence based on a 2-year cut-off.[104] In clinical practice, early recurrent tumours are usually considered true recurrences and are associated with poor survival, while those recurring late are often classified as *de novo* cancers.[105] Compared to primary HCC, early relapsed tumours showed reduced levels of Tregs, increased DCs and infiltrated CD8$^+$ T cells, indicative of an antitumoural TME. However, these CD8$^+$ T cells manifested an innate-like, low cytotoxic phenotype with limited clonal expansion. Furthermore, antigen presentation by DCs to CD8$^+$ T cells was compromised by PD-L1-expressing tumour cells, resulting in a pro-tumourigenic TME.[106] Furthermore, Magen *et al.* highlighted the importance of the T cell and DC interaction in the context of responses to ICIs. PD-1^{hi} progenitor CD8$^+$ T cells were found to engage with CXCL13$^+$ helper T cells, forming cellular triads around DCs enriched in maturation and regulatory molecules; following ICI treatment, differentiation of PD-1^{hi} progenitor CD8$^+$ T cells towards effective anti-tumour CD8$^+$ T cells was regulated by these DCs and CXCL13$^+$ helper T cells.[107] These findings suggest that blockade of the PD-1-PD-L1 axis can restore the crosstalk between DCs and CD8$^+$ T cells, potentially explaining the improved recurrence-free survival of patients treated with adjuvant atezolizumab-bevacizumab following curative-intent resection or ablation in the recent IMbrave050 trial.[108]

As ICIs are gradually introduced in earlier disease stages, the combination regimen of atezolizumab and bevacizumab, targeting PD-L1 and VEGFA, is already well-established in the advanced setting. Despite this, response rates in advanced HCC still hover around 25-30%, underscoring the critical need for predictive biomarkers. Based on single-cell transcriptomics, Cappuyns *et al.* characterised 366,754 intratumoural and peripheral immune cells from patients with advanced HCC treated with atezolizumab-bevacizumab, offering a pivotal impetus for predictive biomarker discovery. Tumours from patients with durable responses were enriched for PD-L1$^+$CXCL10$^+$ macrophages, producing chemokines that attracted peripheral CXCR3$^+$CD8$^+$ effector-memory T cells in the TME. In responders, these CXCR3$^+$CD8$^+$ effector-memory T cells preferentially differentiated into clonally expanded PD-1$^-$CD45RA$^+$ effector-memory CD8$^+$ T cells (CD8$^+$ TEMRA) with pronounced cytotoxicity. Consistent with their patrolling function in responders, CD8$^+$ TEMRA showed a high degree of T-cell receptor sharing with the peripheral blood. These findings not only offer insights into potential mechanisms of response to atezolizumab-bevacizumab in advanced HCC but also provide crucial clues regarding CD8$^+$ TEMRA, PD-L1$^+$CXCL10$^+$ macrophages, or T-cell receptor sharing as potential predictive biomarkers.[109]

Given the intricate immune component within the TME of HCC, it is anticipated that treatment resistance arises from multiple mechanisms. Based on mouse models and single-cell transcriptomics on human HCC, Kan *et al.* demonstrated that increased MZF1 expression in cancer cells resulted in increased PD-L1 ubiquitination, thereby rendering anti-PD-L1 antibodies ineffective.[110] Beyond anti-PD-L1 antibodies like atezolizumab or durvalumab, ICIs blocking PD-1, such as nivolumab, pembrolizumab, or sintilimab are commonly used in the treatment of HCC, often combined with tyrosine kinase inhibitors. Multiple ways for tumours to evade the effect of anti-PD-1-based immunotherapy were hypothesised. Meng *et al.* highlighted the ability of cancer cells to reprogramme CD10$^+$ALPL$^+$ neutrophils, maintaining them in an immature state and thereby

inducing an irreversible exhaustion of T cells.[111] Additionally, Zhou *et al.* found that MAIT cells activated TNFRSF1B on regulatory T cells, promoting immunosuppression and resistance to anti-PD-1 therapy.

Single-cell insights have illuminated the intricate interactions among cancer cells and the innate and adaptive immune system during ICI treatment. However, the insufficient elucidation of the mechanisms underlying ICI refractoriness in HCC remains a notable gap in current knowledge. Identification of reliable biomarkers to predict response or resistance stands as a crucial unmet need, with the potential to revolutionise the treatment landscape of HCC.

Conclusion

Single-cell sequencing techniques have emerged as indispensable tools to unravel the intricate spatiotemporal dynamics within the TME of HCC, providing increased understanding of hepatocarcinogenesis, tumour behaviour and treatment response or resistance. Insights derived from single-cell sequencing experiments have the potential to provide clinicians in the field of HCC with predictive biomarkers, new ICI targets, risk stratification tools and disease activity monitoring (Fig. 3). Nevertheless, before integration into clinical practice, substantial questions remain to be answered. Although the application of single-cell multi-omics in liver cancer research has been limited thus far, it can act as a

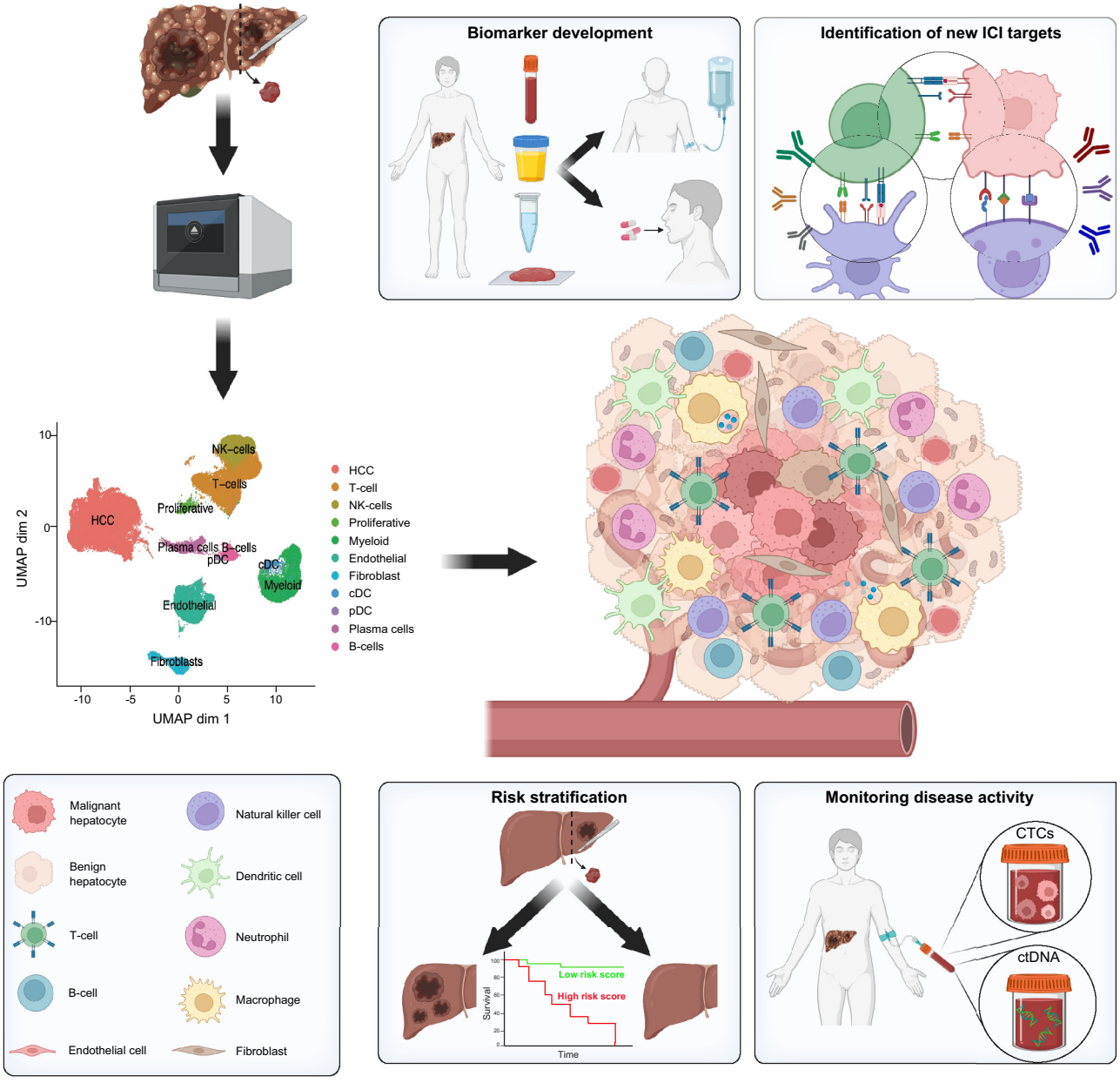

Fig. 3. Potential clinical implications of insights derived from single-cell experiments. UMAP is a two-dimensional technique to visualise the gene expression of individual cells. Clustering is based on the similarity of gene expression profiles. The UMAP in this figure was adopted with consent from Cappuyns *et al.*[109] cDC, classical dendritic cells; ctDNA, circulating tumour DNA; CTC, circulating tumour cells; HCC, hepatocellular carcinoma; ICI, immune checkpoint inhibitor; NK, natural killer; pDC, plasmacytoid dendritic cells; UMAP, uniform manifold approximation and projection.

foundational steppingstone towards biology-driven answers to these critical questions. The introduction of single-cell multi-omics in liver cancer research has the potential to revolutionise personalised medicine in HCC, resulting in improved cost efficiency, reduced unnecessary exposures to potential treatment-related toxicity, and ultimately better patient outcomes.

Abbreviations

CAFs, cancer-associated fibroblasts; cDNA, complementary DNA; CNVs, copy number variations; CSCs, cancer stem cells; CTCs, circulating tumour cells; DCs, dendritic cells; ICIs, immune checkpoint inhibitors; MAIT, mucosal-associated invariant T cells; PLC, primary liver cancer; scRNA-seq, single-cell RNA sequencing; TAMs, tumour-associated macrophages; TME, tumour microenvironment; Tregs, regulatory T cells.

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Conflicts of interest

The authors of this study declare that they do not have any conflict of interest.

Please refer to the accompanying ICMJE disclosure forms for further details.

Authors' contributions

FP: conceptualization & writing-original draft. SC: conceptualization & writing-review and editing. MPG: conceptualization & writing-review and editing. CV: conceptualization & supervision & writing-review and editing. GP: conceptualization & writing-review and editing. DL: conceptualization & supervision & writing-review and editing. JD: conceptualization & supervision & writing-review and editing. All authors read and approved the final manuscript.

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Supplementary data

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Author names in bold designate shared co-first authorship

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