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Review Article

Diverse perspectives to address for the future treatment of heterogeneous hepatocellular carcinoma

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Abstract

Hepatocellular carcinomas (HCCs), which often arise from chronic liver damage, have poor conditional 5-year survival and are recognized as heterogeneous tumors. Considering the heterogeneity of HCCs, diverse perspectives need to be addressed for treating such tumors, besides the findings of conventional imaging modalities and tumor markers. Data from the latest technologies, such as liquid biopsy, and the detection of the presence of cancer cells with stem/progenitor cell markers, gene mutations and diverse pathways, crosstalk with immune cells and cancer-associated fibroblasts, and mechanisms of epithelial−mesenchymal transition provide diverse lines of information. Integration of these data with clinical data might be necessary to develop effective therapies for precision medicine. Here, we review several aspects of dealing with the complexity of heterogeneous HCCs.

Keywords: Cancer research, Medicine

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1. Introduction

Liver cancers, most of which are hepatocellular carcinomas (HCCs), constitute the second most common cause of death from cancer worldwide [1]. For liver cancer, the conditional 5-year survival (a survival estimate based on the data of patients who have survived for one or more years) is very poor and does not increase, suggesting a high rate of recurrence or appearance of new lesions of liver cancers [2]. HBV or HCV infection, alcoholic liver disease, and non-alcoholic steatohepatitis are representative causes of hepatocellular carcinoma [3]. Chronic liver damage induces cell necrosis and affects the cell regeneration cycle. This can cause cancer by the introduction of chromosomal instability, which alters the functions of immune cells, fibroblasts, etc. [4] Clinically, HCC is diagnosed by tumor markers such as α-fetoprotein (AFP), lectin-reactive AFP (AFP-L3) [5, 6, 7], and des-γ-carboxy prothrombin (DCP) [8, 9, 10] and imaging modalities such as ultrasonography, computed tomography, and magnetic resonance imaging [11]. However, like gastric cancer, HCC is not always diagnosed by biopsy. Recently, liquid biopsies, which detect circulating tumor cells and microRNAs (miRNAs), have been attempted as alternative non-invasive diagnostic methods [12, 13, 14, 15, 16, 17].

The treatment approach for HCC is determined by many factors, such as liver function, tumor malignant behavior, tumor number, tumor size, lesion, and patient age. However, surgical retrieval of HCC tissues is restricted to patients with good liver function and patients undergoing liver transplantation. Treatment of HCC without resection or transplantation, such as by radiofrequency ablation therapy (RFA); microwave coagulation therapy; transcatheter arterial infusion; transcatheter arterial (chromo) embolization (TACE, TAE); radiation therapy; and molecular targeted therapy by sorafenib, regorafenib, and lenvatinib [18], are selected according to the above tumor factors and patient factors [19, 20]. In addition, immune checkpoint inhibitors are expected to be implemented as a new approach to HCC treatment.

HCC can lead to various etiologies and variable patient conditions and are therefore treated by different approaches. Furthermore, the heterogeneity of HCCs is well established not only among patients and tumors but also within tumors. Recent advances in the analysis of genome mutational signatures or analysis of cancer stem cells (CSCs), which revealed differences in morphology and malignant potential, have been elucidating the complex features of this heterogeneity [21, 22]. Furthermore, HCC is affected by factors such as immune cells [23], cancer-associated fibroblasts (CAFs) [24], and stress caused by treatment (Fig. 1). All these factors contribute to the heterogeneity of HCCs. Additionally, epithelial−mesenchymal transition is a well-known phenomenon that occurs in the early steps of metastasis [25]. Thus, understanding these factors is essential for selecting effective treatment approaches for HCC and providing insights into future precision medicine. Here,

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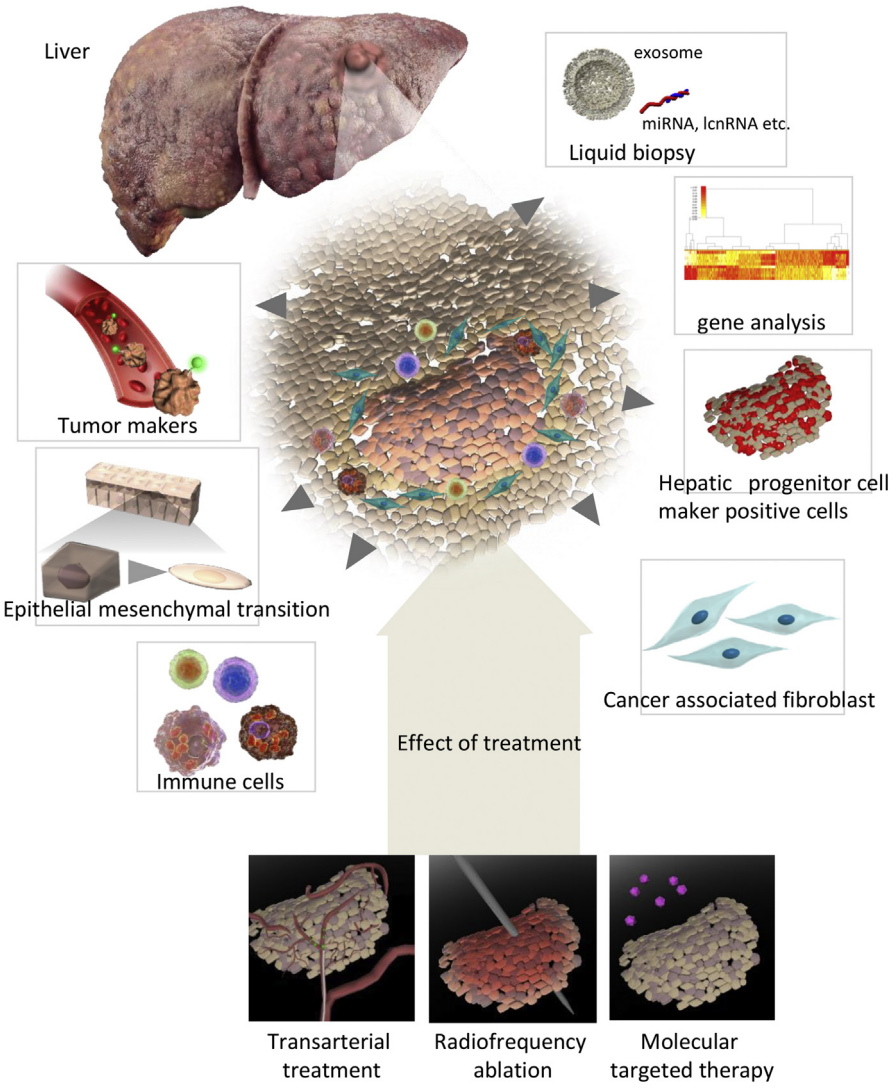

Fig. 1. Overview of heterogeneous hepatocellular carcinoma, underlying factors, and treatment. Data of tumor markers, liquid biopsy, gene mutations, and HPC markers, in concert with information on immune cells, CAFs, and EMT, might be necessary to select the appropriate treatment in the future.

HCCs are reviewed from several aspects to establish future precision medicine for this cancer type.

2. Main text

2.1. Serum tumor markers

Serum tumor markers are widely used to detect HCC clinically. The classic and most popular tumor marker is AFP. AFP can be detected not only in HCC but also chronic liver disease. Several investigators have reported that measuring the *Lens culinaris* agglutinin reaction fraction of AFP (AFP-L3) is useful to distinguish between non-

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neoplastic liver diseases and HCC. Recently, AFP-L3 was reported to be a marker of HCC with malignant behavior (such as portal vein invasion), lower tumor classification, and advanced tumor stage, regardless of small tumor size and/or lower serum AFP concentration. Patients with elevated levels of AFP-L3 have a lower survival rate than those of patients with lower AFP-L3 levels [6]. Therefore, Tamura *et al* reported that owing to the high recurrence and poor prognosis of HCC patients with AFP-L3 concentrations of >15%, they should be treated with careful consideration [26]. We recently confirmed by a retrospective study that patients positive for more than two hepatic progenitor cell (HPC) markers tend to have high AFP-L3 levels [27]. In addition, a highly sensitive immunoassay using on-chip electrokinetic reaction and separation by affinity electrophoresis (micro-total analysis system; μTAS) for AFP-L3 has been developed, and AFP-L3 frequency can now be measured accurately at very low AFP concentrations (from 2 ng/mL). This highly sensitive AFP-L3 is more sensitive than conventional AFP-L3 for discriminating HCC, particularly in early-stage HCC and subgroups with lower AFP concentrations [28, 29]. Furthermore, we recently encountered cases in which only increased levels of the highly sensitive AFP-L3 were detected before the detection of HCC recurrence by imaging modalities, suggesting that AFP-L3 can be employed for the early detection of HCC recurrence after potentially curative treatment [30]. DCP, also known as protein induced by vitamin K absence II (PIVKA-II), has been reported to be a useful marker and predictor of HCC [9]. DCP differs from prothrombin in the composition of amino acid residues and was initially discovered in HCC patients in 1984 [8]. The mechanisms of DCP production by the tumor are not yet fully understood; however, DCP does not always parallel the behavior of AFP and AFP-L3, suggesting the complex heterogeneity of HCC. The combination of the measurement of these three tumor markers is useful for early detection and during follow-up examinations. Nonetheless, although these tumor markers may be related to the malignant behavior of HCC, the specific molecular targeted therapies, chemotherapies, or immune therapies are not determined only by tumor markers.

2.2. Liquid biopsy

Apart from the conventional tumor markers AFP, AFP-L3, and DCP, new methods to detect the existence of HCC or early metastasis, such as liquid biopsy, are being performed by detecting circulating cells and cell-free nucleic acids. These methods are currently not widely used clinically but are expected to facilitate more sensitive diagnosis and enable better decision making by unraveling genetic and epigenetic aberrations that reflect the characteristics of HCC. Regarding the circulating cells, physical methods such as separation of cells by density, size, migratory capacity, deformability, and electric charge, as well as biological methods that mainly rely on antigen−antibody binding and antibodies against tumor-specific biomarkers such as epithelial cell adhesion molecule (EpCAM), are in use. "Cell-search^{TM}" and

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"CTC-chip" are frequently used to detect cancer cells from the peripheral blood [14]. Although EpCAM is the most popular stem/progenitor cell marker of HCC, this technique is limited by the detection of only some parts of HCC. Regarding cell-free nucleic acids, circulating cell-free DNA and RNA (cfDNA and cfRNA), of which miRNA and long non-coding RNA (lncRNA) are popular, are being increasingly analyzed. The miRNA and lncRNA are packed into small membrane vesicles called exosomes and stably detected in the plasma and serum. A large number of miRNAs and lcnRNAs are reported to be related to HCC, some of which are the subjects of attempts for use in combination with conventional tumor markers to improve diagnostic accuracy in the future. Accumulating evidence of circulating cfDNA and cfRNA will clarify their clinical utility as not only markers for detecting HCC but also prognostic markers [12, 13, 14, 15, 16, 17].

2.3. Stem/progenitor cell markers

The existence of CSCs or tumor-initiating cells has been widely discussed, and CSCs have been actively investigated as a target of effective cancer treatment. CSCs have been reported to have high metastatic ability and radiotherapy/chemotherapy resistance. The origin of CSCs is also controversial [4, 31]. CSC hierarchical models are well known; however, the theories remain disputed. The transdifferentiation and retrodifferentiation by genetic/epigenetic events caused by chronic inflammation such as fibrosis, extracellular matrix remodeling, mechanical stress, hypoxia, acidosis, metabolic change, effect of immune cells, and treatment such as chemotherapy underlie the highly heterogeneous HCC populations. Irrespective of the cell origin, these cells are currently being detected by the expression of HPC markers such as EpCAM [32, 33], CD44 [34], CD133 [35], CD13 [36], CD24 [37, 38], neural cell adhesion molecule (NCAM) [39, 40, 41], cytokeratin 19 (CK19) [42], delta-like 1 homolog (DLK1) [43], and sal-like protein 4 (SALL4) [44]. The frequencies of cells positive for these markers are analyzed by immunohistochemistry and real-time PCR. From previous reports, around 10% of operated HCC patients express each marker; however, the frequency of HCC with more than two HPC markers, the signals influencing the expression of these markers, and the complex heterogeneity detected by a combination of HPC markers are aspects not sufficiently understood yet. Of all these HPC markers, EpCAM is the most popular. It is reported to be associated with poorly differentiated HCC and high serum levels of AFP [33]. Although accumulating evidence shows that HCCs positive for these markers have malignant behavior and poor prognosis, at present, these markers cannot be predicted from clinical data, and their association with genetic/epigenetic events is unclear. Recently, we reported a retrospective study that by using 251 operated HCC tissues and immunostaining for four HPC markers, 18.3%, 7.1%, 14.3%, and 8.0% of patients were found to have high levels of DLK1, NCAM, EpCAM, and CK19 in tumors, respectively. The expression of two or more HPC markers was a

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significant predictor of poor HCC outcome, and serum levels of AFP/AFP-L3 correlated with the expression of HPC proteins. However, HPC marker-targeted therapy is not established yet [27]. Further mechanistic analysis of these markers and association with clinical data and genetic/epigenetic information will be needed for clinical use. Recently, these markers have also been employed to detect the above-mentioned circulating tumor cells by liquid biopsy. Thus, these markers are expected to not only predict tumor behavior but also determine treatment strategies.

2.4. Gene mutations and driver pathways

HCCs usually occur in a background of chronic liver injury, which results in acceleration of the cell damage−regeneration cycle and development of chromosome instability. Genetic and epigenetic alterations gradually accumulate in these chronic inflammatory circumstances. In HBV infection, oncogenic properties of the HBx protein and insertional mutagenesis of the HBV genome in cancer genes have been reported. Each HCC genome is the result of a unique combination of somatic gene alterations, which might cause the complex heterogeneity of HCCs. The most frequent mutations observed in HCC are in the *TERT* promoter, followed by *TP53* and *CTNNB1*. In addition, low-frequency mutations in genes such as *AXIN1*, *ARID2*, and *ARID1A* are observed [45]. Integration of mutation data with recent whole-genome sequencing has identified the major pathways that are potential targets for HCC treatment: (1) telomere maintenance, (2) Wnt/β-catenin pathway, (3) P53 cell cycle pathway, (4) epigenetic modifier, (5) oxidative stress pathways, and (6) PI3K/AKT/MTOR and RAS/RAF/mitogen-activated protein kinase pathways [45]. It is ideal to use this information for selecting the appropriate therapy; however, it is currently not widely analyzed clinically, and no therapy has been identified on the basis of the information from these mutated genes and driver pathways. In addition, it is not clear if this pathway analysis is indeed applicable for advanced-stage, complex, heterogeneous HCC in terms of treatment decision making. Recently, multiple genome platform data such as the exome sequence, copy number, mRNA sequence, miRNA sequence, methylomics, and proteomics were analyzed in an integrated manner for overcoming these complex problems for clinical applications [1]. Combining the above information and clinical information may reveal some critical aspects of personalized medicine. Liver cancer cell biopsy is not usually performed, but in the future it may be necessary to select the appropriate treatment.

2.5. Immune cells

One of the functions of the immune system is to exclude cancer cells. At the early stages of HCC, cancer cells are excluded under the surveillance of natural killer cells and cytotoxic T cells (CTLs). During the development of cancer, an immunosuppressive microenvironment against cancer cells develops by the cytokines,

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chemokines, growth factors, and metabolites released by immune cells and cancer cells. Immune cells that compose this immunosuppressive microenvironment include regulatory T cells (Tregs), tumor-associated macrophages (TAMs), and myeloid-derived suppressor cells [46]. CAFs also participate in the immunosuppressive microenvironment. Clinical trials for HCC are evaluating the antibodies against programmed cell death 1 (PD-1), programmed cell death-ligand 1 (PD-L1), and CTL-associated antigen 4 (CTLA-4), which are considered immune checkpoint inhibitors, for use in monotherapy or combination therapy. The concept of these therapies is groundbreaking and can change the usual treatment strategies. Patients responding to some immune checkpoint inhibitors are showing favorable results. Immune checkpoint inhibitors in combination with existing loco-regional therapy such as TACE and RFA or molecular targeted agents are expected to improve patient prognosis [18, 47]. Remaining challenges include the assessment of which patients or what kinds of HCC respond to the immune checkpoint inhibitors, countermeasures to adverse effects, and cost. Recent papers have reported additional basic mechanisms of immune cells using animal models. Wan *et al* reported that TAMs produce interleukin-6 (IL-6), which promotes CSCs and tumorigenesis [48]. Ma *et al* put forth an interesting point of view that the dysregulation of lipid metabolism in non-alcoholic fatty liver disease (NAFLD) causes a selective loss of intrahepatic CD4+ T lymphocytes, which have abundant mitochondria and produce high levels of reactive oxygen species, but not of CD8+ T lymphocytes. This dysregulation accelerates hepatocarcinogenesis, suggesting the importance of lipid regulation and immune systems in tumorigenesis [49]. Furthermore, Shalapour *et al* reported that liver resident immunoglobulin A-producing cells are accumulated in NAFLD. These cells express PD-L1 and IL-10 and suppress liver cytotoxic CD8+ T lymphocytes, which prevents the emergence of HCC and causes the expression of a limited repertoire of T-cell receptors against tumor-associated antigens [50]. All these findings suggest that an understanding of the changes in immune system functions in HCC is essential for effective HCC prevention and treatment. Further basic studies may be necessary to develop more effective immune therapies.

2.6. CAFs

Fibroblasts near cancer cells, known as CAFs, also crosstalk with cancer cells and affect proliferation, motility, drug resistance, EMT, immunosuppressive effects, and angiogenesis; most of these effects are still the subject of basic research but nonetheless potentially critical components to consider. CAFs are reported to differ from non-tumor fibroblasts in terms of abundant mRNA expression of αSma, DNA methylation-based epigenetic changes, and collagen 11A1 production. CAFs produce many cytokines; chemokines; and growth factors, such as stromal cell growth factor (SDF-1), hepatocyte growth factor (HGF), epidermal growth factor (EGF), fibroblast growth factor (FGF), Wnt families, and IL-6. These factors stimulate

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cancer cell growth directly or indirectly. Transforming growth factor (TGF)-β and platelet-derived growth factor (PDGF) signaling affects EMT. CAFs also affect immunosuppressive effects by inducing Tregs, tumor-associated neutrophils, and TAMs, of which the latter are polarized toward the anti-inflammatory phenotype M2. Furthermore, CAFs secrete angiogenic factors such as vascular endothelial growth factor (VEGF), PDGF, and matrix metalloproteinases, thereby promoting angiogenesis [24, 48]. Exosomes containing abundant miRNA have also been implicated in this crosstalk recently. Fang *et al* reported that HCCs secrete miR-1247-3p and convert normal fibroblasts to CAFs, which promote cancer progression by secreting pro-inflammatory cytokines such as IL-6 and IL-8 [51]. Meanwhile, CAFs also produce exosomes such as miR320a, which influence antitumor effects [52]. These findings highlight the potential of CAFs as anticancer therapy targets.

2.7. The effect of treatment and EMT transition

In cancer progression, EMT plays a crucial role in the early steps of metastasis, when cells lose cell−cell contact owing to the decrease in E-cadherin and acquire increased motility to spread into surrounding or distant tissues. These events remain the subject of basic research; however, they are undoubtedly important in tumor progression. A strong inducer of EMT is TGF-β, which can orchestrate both fibrogenesis and carcinogenesis, showing rising cytokine levels in cirrhosis and late-stage HCC. miRNA also causes EMT during HCC progression. Besides factors like immune cells and CAFs, the type of treatment can also affect the fate of HCC. EMT can occur in fibrosis and cancer, as well as during HCC treatment. Tong *et al* reported that RFA can cause EMT. RFA can lead to the formation of a transition zone between normal liver tissues and necrotic coagulation, where the residual cancer cells are exposed to a hypoxic condition. They cultured HCC cells under heat treatment and hypoxic conditions to mimic the post-RFA condition and demonstrated that the hypoxic HCC cells changed to mesenchymal morphology and gained more invasive, metastatic, and chemo-resistance potential compared to the control. They mentioned that hypoxia-inducible factor (HIF)-1α contributed to this step [53]. Huang *et al* reported the same phenomenon by HIF-1α after TACE treatment [32]. However, Dong *et al* reported that sorafenib suppresses the EMT of HCC after insufficient RFA therapy [54]. Together, these results indicate that both the tumor environment and HCC treatment strategy regulate the EMT, and when possible, curative therapy is ideal to prevent EMT.

3. Conclusions

The development of new technologies is gradually clarifying the complex heterogeneity of HCCs and the mechanisms underlying this heterogeneity. Furthermore, molecular targeted therapies and immune checkpoint inhibitors present new

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possibilities for treatment of HCCs. Given that HCCs are highly heterogeneous, often interacting with various factors, curative approaches such as surgery might be ideal if the liver function permits. Without surgery, if recurrence is to be expected, combination therapy might be more effective than monotherapy to prevent recurrence. If clinical information including patient characteristics such as imaging modality data, tumor marker data, and effective treatment data are used in conjunction with liquid biopsy data, progenitor cell marker data, immune cell data, CAF data, and gene mutation data [55], an effective therapeutic approach might be identified and chosen for personalized medicine in future.

Declarations

Author contribution statement

All authors listed have significantly contributed to the development and the writing of this article.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

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