Hepatocellular Carcinoma: Understanding Molecular Mechanisms for Defining Potential Clinical Modalities

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Introduction

Liver cancer is at sixth position in cancer incidence globally and accounts for 8.2% of total cancer deaths. The different categories of primary liver cancer are intrahepatic cholangiocarcinoma (ICC), hepatocellular carcinoma (HCC), fibrolamellar carcinoma, hepatoblastoma. These categories have distinct changes in their molecular, histological, and pathological features. HCC alone accounts for 85-90% of liver cancer cases[1]. Almost 2/3 of the population affected by HCC is found in East Asian and south-east Asian countries, making this disease endemic to the region[2]. Globally, 5-year median survival is below 20% in hepatocellular carcinoma[3]. Major risk factors for HCC include chronic infection of hepatitis B virus & hepatitis C virus, excessive consumption of alcohol, exposure to aflatoxin, physiological state such as non-alcoholic fatty liver disease, and diabetes[4]. According to the Barcelona Clinic Cancer Liver Classification (BCLC) algorithm, curative care for HCC involves tumor resection, ablation, and liver transplantation[5]. However, this mode of treatment is offered to patients diagnosed in an early stage of the disease. Current research suggests that only 20% of patients are diagnosed in the early stage[6]. The lacunae in diagnosis are the unavailability of promising liquid-based biomarkers and detection limits of scanning techniques. Palliative care involving chemo/radiation-based treatment is given to patients with intermediate and advance stage disease. Out of this, 70% of patients come back with a relapse of disease and suffer from side effects of treatment[7,8].

A new approach should be considered to identify diagnostic markers and achieve better therapy response to overcome disease management challenges. Recent advances in the omics field shed light on the pathogenesis and molecular classification of HCC[9–11]. The omics approach can help to investigate new markers to improve the therapeutic outcome. Liver carcinogenesis involves both genetic and epigenetic changes. It is impossible to target all genetic variations due to tumor heterogeneity, but gene signature can be manipulated as epigenetic changes are reversible[12]. Therefore, epi-drug-based treatment may act as an alternate treatment strategy instead of targeting a single protein or molecular pathway. Epi-drugs can be beneficial not only for the treatment of HCC but also for dealing with cancer resistance[13,14].

This article focuses on the existing approach in diagnosis and treatment for the management of HCC. We also review transcriptomic-based signatures of HCC for patient sub-categorization and having potential implications for diagnosis and therapy. Finally, we propose an epi-drug-based treatment strategy based on the epigenetic landscape of HCC.

Diagnosis of liver cancer

Five standard WHO-approved guidelines include the European Association for the Study of Liver Disease (EASL)[15], American Association for the Study of Liver Diseases (AASLD)[16], Asia-Pacific Association Study of the Liver (APASL) [17], EASL-EORTC Clinical Practice Guidelines[18], and the updated AASLD guidelines are used for diagnosis of liver cancer. The diagnosis is primarily based on imaging techniques such as ultrasound, CT scan, and conventional MRI[19]. Invasive biopsies are not helpful for the diagnosis of liver tumors. The myriad of risk factors involved in using biopsy is the local spread of HCC along the needle track and different complications observed in individual

patients[20]. The early-stage diagnosis of hepatocellular carcinoma continues to be crucial due to lacunae in sensitivity and specificity of the diagnostic methods, due to which an ample number of tumors go undetected. The complete list of diagnostic methods with detection limit has been enlisted in table no. 1. The various factors responsible for undetectable tumors involve a lack of specific markers and asymptomatic condition during the early stages of HCC[21]. Thus, the diagnosis of tumor befall is well spread and has reached the advanced stage of the tumor.

The diagnostic marker used most prominently is serum α-fetoprotein (AFP)[22]. AFP level increases beyond 20 ng/mL in more than 70% of patients with HCC. However, AFP elevations are not explicitly associated with HCC as AFP levels from 10-500 ng/mL and even occasionally to 1000 ng/mL may be seen in patients with a high degree of necro-inflammatory activity like chronic viral hepatitis[23]. Chan *et al.* in 2008 have shown AFP could be better used as a prognostic marker to evaluate response to treatment and detecting recurrence instead of diagnosis[24,25]. Studies have shown that multiple combinations of markers provide more appropriate results in diagnosis than a single marker. A recent study has investigated the use of HSP90α(Heat shock protein 90) combined with AFP and TK1(Thymidine kinase 1) to diagnose hepatocellular carcinoma with more efficiency[26]. A study from Beijing YouAn Hospital found that for early diagnosis of HBV-related HCC, a combination of AFP, GPC3, and GP73 had the highest diagnostic value[27]. Gosh *et al.* have shown that the exosome encapsulated microRNAs could be used as a circulating diagnostic marker for hepatocellular carcinoma with low AFP levels[28].

Another marker, α-L-fucosidase (AFU), is expressed in liver cirrhosis patients[29]. However, limited research is available about the utility of AFU in the diagnosis of HCC. In the liver and gall bladder, cell membrane protein 5'-

Nucleotidase (5'-NT) is released into the blood during hepatic injury or obstruction[30]. It has been observed that 5'-NT levels also increase with age and during pregnancy[31]. Other markers such as AFP-L3, GPC-3(glypican-3), and Descarboxy prothrombin (DCP) also show inconsistent data due to low sensitivity and specificity. Hence, the discovery of putative liquid biomarkers is required, which can associate with the tumor progression, recurrence, and effectiveness of therapeutic programs.

Treatment regime and Limitations of chemotherapy in liver cancer

Treatment for HCC is decided based on different stages of tumor detection[32]. BCLC algorithm is widely used for treatment as it considers tumor stage, liver function, performance status, and treatment impact (Figure 1). Early-stage cases are treated with surgery, ablation, or liver transplantation. The patient undergoing surgery showed 70% recurrence within five years[33]. The currently used methods for tumor ablation in HCC are percutaneous ethanol injection (PEI) and radiofrequency ablation (RFA). PEI consists of the direct injection of absolute ethanol into HCC nodules[34]. RFA is responsive for a tumor with >4cm in size. It involves necrosis of tumor using a needle tip electrode that reaches temperature up to 100°C[35]. Microwave ablation and irreversible electroporation have shown more promising results than tumor removal with PEI[36].

Patients with an intermediate stage having a tumor size greater than 5cm or multinodular HCC with no vascular invasion are treated with trans-arterial chemoembolization (TACE). TACE is used to obstruct the nutrient supply to the tumor using the occlusion of arterial blood vessels[37]. Chemotherapeutic drugs such as doxorubicin or cisplatin are given during embolization, allowing prolonged exposure of the drug to tumor cells, resulting in tumor reduction. Yeo W *et al.* showed that the overall response rate for doxorubicin-treated patients

was 10.5%. Moreover, alone doxorubicin and PIAF combination had no significant difference in response rate but showed treatment-associated toxicity in patients[38]. Another study showed that combinatorial treatment of fluorouracil, leucovorin, and oxaliplatin failed in improving survival compared to doxorubicin[39]. In a multicohort study involving patients with unresectable tumors treated with TACE, overall survival was approximately 26-40 months, with only 52% of patients receiving treatment benefits[40,41]. In some cases, selective internal radiation therapy (SIRT) is used in patients with intermediate-stage HCC. Intraarterial infusion of radioisotope labeled microspheres is carried out in this modality. Another radiation-based technique known as stereotactic body radiation is used for patients with >3cm of the tumor.

Systemic chemotherapy is given for advanced stages of HCC. NCCN Clinical Practice Guidelines in Oncology (NCCN Guidelines) have recommended sorafenib and lenvatinib as the first-line of systemic therapy for patients with unresectable HCC[42]. Brivanib, sunitinib, erlotinib, and regorafenib are other preferred drugs for late-stage HCC treatment. Kudo et al. observed that treatment lenvatinib results in significantly higher overall survival than sorafenib and improvement in all secondary efficacy endpoints. This trial further results in FDA approval of lenvatinib as the first line of therapy for HCC[43]. Sorafenib and sunitinib are protein kinase inhibitors targeting VEGFR, PDGFR, and Raf kinase pathway. However, a study suggested that sunitinib showed an adverse effect on the patients and had no advantage over sorafenib[44]. Moreover, sorafenib has been explored extensively in the systemic treatment of advanced stage HCC and combination with TACE, but it provided contradictory results[45,46]. Brivanib is an inhibitor of FGF1 and VEGFR2. Phase II clinical trials of brivanib showed the ineffectiveness of the

drug compared to sorafenib for improving overall survival[47,48]. EGFR inhibitor erlotinib or cetuximab was in phase II clinical trials of advanced stages of HCC. However, the trial results did not show the anti-tumor effect of cetuximab in HCC patients[49]. Interestingly, erlotinib showed a positive response in treatment by increasing OS to 13 months and a response rate of 59%[50].

As discussed earlier, ablation treatment is possible in less than 40% of patients due to late diagnosis, and only 20% are treated with TACE. For the patients in advance stages of HCC, treatment modalities are limited to systemic therapy, and response rates are also significantly less due to resistance towards available chemotherapy. The multimodal treatment involving more than one therapeutic drug has also failed in different combinations due to cytotoxicity and poor trial outcomes. Despite the significant research in targeted therapy of HCC management, a promising drug is yet to be employed. Thus, the hunt for combinatorial treatment with different therapeutic agents continues.

Molecular landscape of liver tumor tissue for patient stratification and identification of alternate targets.

Over the past years, HCC classification has majorly focused on histological analysis of tumor tissues. However, molecular profile and clinical attributes have a significant impact on the prognosis of the disease, thereby redefining HCC into several subgroups. Boyault *et al.* published molecular classification systems for HCC composed of 6 groups[51]. The groups were based on mutation profile, disease prognosis, and transcription landscape. The first group has patients with hepatitis B infection and low viral load, increased AFP levels, and high IGF2 expression, whereas the second group has a high viral titer and has an association with MVI and satellitosis. However, the difference

in groups 3 and 4 is based on histological parameters. The third group consists of poorly differentiated tumors having the worst prognosis; on the other hand, group 4 has well-differentiated tumors. Group 5 and 6 have a low proliferation rate and activated wnt-signaling pathway. Moreover, pathways are differentially activated in different groups. Another group classified HCC into three groups based on histology and expression analysis of tumor[52]. In this study, the first group showed the presence of satellitosis and MVI. Group 2 has high AFP expression. 3rd group consists of the well-differentiated tumor having a low proliferation rate.

Tumor morphology-based classification has been proposed by Murakata *et al.* [53]. The nodal status of the tumor was correlated with survival and recurrence of the disease. Moreover, the miRNA profile of HCC patients has been used to classify the sorafenib responders[54]. *c-myc* signaling and EB-1 protein were functionally linked with HCC[55]. Similar findings were observed by Lee *et al.* in progenitor-like HCC, which correlates with poor prognosis[56]. In another study, HCC progenitor-like signature consisting of CK-19, Ep-CAM, and CD133 was seen by Woo *et al.*[57]. Morofuji *et al.* found gene signature of early recurrent HCC, including ERK1, PKG, Apaf1, and Bcl-X. Further, ERK1 and Bcl-X were identified as genes associated with the poor prognosis of HCC[58]. However, these studies do not consider the survival status of an individual while proposing subtypes.

Jiang et al. showed that heterogeneity exists in proteomic profiling of paired early-stage HCC patients[59]. The tumors were segregated into three subtypes: S-I, S-II, and S-III. S-I tumors have increased expression of liver-associated functional proteins. In contrast, S-II and S-III have more proliferative nature due to overexpression of cell-cycle-related proteins. Furthermore, S-III were more aggressive and had a high expression of KRT19 and MMP9, associated

with poor prognosis. Gao et al. sub-grouped 159 HBV infected patients based on survival, tumor thrombus, and multi-omics profile[60]. These sub-groups were classified based on metabolic rewiring, alterations in the microenvironment, and cellular proliferation. Moreover, the study proposed two prognostic markers PYCR2 and ADH1A.

In the past decade, data generated under the TCGA consortium can be used to understand the gene expression profile of patients and obtain correlation with clinical attributes[9]. Machine learning algorithms are necessary to analyze such kinds of multivariate data. The molecular alterations obtained from the TCGA-LIHC cohort (423 patients) can be explored to predict new targets and rationalize the combinatorial therapy. Transcriptome data generated from TCGA-LIHC identified over 13000 differentially expressed genes compared to cut-margin samples, and around 3330 genes correlated with poor survival (pvalue<0.05). Furthermore, 1730 genes have overlap between the DE gene list and gene correlated with patient survival. The majority of overlapped genes showed more than 30% alteration compared to adjacent normal in this cohort and had a significant association with OS. Patients were categorized into different groups using clustering analysis of gene expression. It was observed that these genes belong to metabolism-related pathways and cellular proliferation-related family (Figure 3). Deep learning computational framework on the TCGA-LIHC dataset suggested that aggressive subtype has TP53 inactivation with high expression of KRT10, EPCAM, and active AKT, WNT signaling [61]. Furthermore, drugs and small molecular compounds are available to target these genes. Schulze *et al.* reported that potential gene targets have FDA-approved drugs in 28% of liver tumors [62]. Therefore, these genes can be taken for the prognosis of the disease, and targeting them may improve patient survival.

Gene expression analysis of liver cancer samples can also be utilized to identify new markers for diagnostic purposes. For example, SPP2 is downregulated at the transcript level in the case of HCC. This gene is deregulated in multiple HCC cohorts. Moreover, a stage-wise decrease at transcript level was observed in HCC TCGA data. Also, the downregulation of SPP2 leads to a significant decrease in patient survival (Figure 4). This observation indicates that SPP2 level is associated with normal liver function, and change in levels can be a measure of liver carcinogenesis.

Epi-drug based treatment for improvement of therapeutic outcome

The lack of success in disease management can be explained by the multifactorial nature of carcinogenesis involving multiple mutations and global level epigenome alterations[63–65]. Epigenetic changes being reversible can be useful to understand the relationship between tumor biology and help in redefining therapeutic response[12]. Epigenetics deals with changes in gene expression without change in the DNA sequences[66]. Despite all cells having the same DNA sequence, epigenome decides cell fate regarding differentiation, cell proliferation, and cell death[67,68]. The widely studied epigenetic marks are DNA methylation, histone post-translational modifications, and non-coding RNAs. DNA methylation is the most characterized heritable epigenetic mark. Herein a methyl group is transferred onto the cytosine of the CpG di-nucleotide-rich region in the DNA by DNMT enzymes[69]. DNA methylation plays a vital role in gene inactivation, genomic imprinting, attaining tissue-specific gene expression, and X chromosome inactivation[69].

Similar to DNA modification, histone proteins also undergo post-translational modifications carried out by chromatin modifiers, namely writers, readers, and erasers[70]. The well-studied modifications include methylation, acetylation, phosphorylation, and ubiquitination. Histone methylation involves the addition

of methyl group at the lysine or arginine residue on the protruding histone tails. Histone methylation marks can result in repression of transcription or gene activation[71]. A typical example of gene suppression is trimethylation at H3K9, H3K27 whereas methylation at H3K4, H3K36, and H3K79 enhance transcriptional activity[71]. Histone acetylation is the transfer of acetyl group from Acetyl CoA. This reaction leads to a change in electrostatic interaction between DNA and histones, resulting in the unwinding of chromatin and enhances gene transcription[72]. Histone phosphorylation has an essential role in DNA damage repair, gene transcription, and chromatin condensation during mitosis[72]. Illustration for chromatin-associated modifications and the role of epigenetic modifiers are shown in Figure 5. Non-coding RNAs are the transcribed intragenic regions of the DNA that are not translated into proteins. These entities govern gene silencing via RISC and RNA-induced transcriptional silencing (RITS) complex formation[73].

Different research groups have extensively studied the epigenetic landscape of liver carcinogenesis. Moreover, in the past few years, researchers are investigating the epigenetic basis of chemoresistance in HCC. *Lie et al.* showed that lysine-specific demethylase 1 (LSD1) is upregulated in LGR5+ cells contributing to stemness and chemoresistant property. Mechanistically, LSD1 removes the H3K4 methylation mark from the promoter of genes which inhibit Wnt-signaling. Thus, promoting pathway activation, which is essential for stemness and chemoresistance[74]. EpCAM+ liver cancer cells have a high expression of CHD4(Chromodomain helicase DNA binding protein), a DNA damage response protein. The abundance of CHD4 in liver cancer cells leads to epirubicin resistance[75]. Zinc-fingers and homeoboxes 2 (ZHX2) is one of the signature proteins which is downregulated in liver CSCs and associated with tumor progression. It has been found that low expression of ZHX2 is correlated

with epigenetic regulation of OCT4, SOX4, and NANOG by H3K36 methylation[76]. Oriana Lo Re *et al.* observed that low expression of MacroH2A1 leads to paracrine mediated chemoresistance and imparts CSCs property to the tumor cell[77]. Another study showed that the regulator of chromosome condensation 2 (RCC2) promotes metastasis and cisplatin resistance in HCC[78]. Ling *et al.* discovered that USP22 helps to attain chemoresistance by hypoxia-driven p53 mutant tumors[79]. Hypoxia-induced expression of carbonyl reductase1 leading to chemoresistance in HCC was seen by Tak *et al.* [80]. H19 lncRNA has been shown to sensitize sorafenib or doxorubicin-resistant liver cancer cells[81]. lncRNA CRNDE has been seen to interact with histone methyltransferase to enhance their effect of inhibition of tumor suppressors and induce resistance in tumor cells[82].

Epigenetic alterations can be targeted by the class of small-molecule inhibitors that specifically inhibit or revert the changes[83]. This class of inhibitors are referred to as epi-drugs. Different research groups have synthesized Epi-drugs for all the three prominent families of epigenetic modifiers- readers, writers, and erasers. Many epi-drugs have cleared pre-clinical trials, and initial phase trials have shown promising results. Few epi-drugs are clinically approved for the treatment of hematological malignancies. In some studies, treatment of epi-drug in solid tumors helps in sensitizing tumor cells to chemotherapy[84,85]. These findings have promoted the research on inhibitors of HDAC, HAT, and DNMTs in combination with chemotherapeutic drugs. In HCC and gastric cancer, the inactive or suppressed state of tumor suppressor genes (TSGs) is mainly attributed to the overexpression of DNMTs and HDACs, leading to heterochromatinization. The reversion of chromatin state using epi-drugs further leads to activation of TSG and restrains tumor growth[86]. Ongoing pre-clinical trials have been carried out with HDAC and DNMT inhibitors in

combination or in comparison with each other to study the anti-tumor effects of drugs. Guadecitabine (SGI-110), a DNMT inhibitor with sorafenib and oxaliplatin, is in phase II clinical trials for HCC (NCT01752933). Multicentre phase I/II of clinical trials using belinostat (HDAC inhibitor) in the patients with unresectable HCC showed tumor stabilization effect[87]. A study showed that the combination of Panobinostat and sorafenib significantly decreased tumor volume by inducing apoptosis in the tumor[88]. A group of researchers observed that DNMT inhibitor 5'-Aza-2'deoxycytidine and HDAC inhibitor SAHA down-regulates DNMT1, DNMT3a, DNMT3b, and HDAC1 and upregulates GSTP1 and SOCS1 gene expression, which further result in inhibition of cell viability and induced apoptosis[89]. A detailed list of potential epi-drugs is given in table no (2). These findings indicate the ability of epi-drugs, which can restructure treatment strategy for HCC.

Future perspectives

The most effective way of controlling HCC cases is preventing the disease by spreading knowledge of etiological agents and hepatitis B vaccination. An increase in surveillance is one of the strategies to achieve better survival. This practice helps in the early diagnosis of HCC and monitors progression-free survival, and develops the quality of life.

Diagnosis of HCC at an early stage is crucial to start treatment at the right time and improve patient survival. Due to less sensitivity of current diagnostic techniques, ultrasound scanning of high-risk individuals should carry out every three months. Although ultrasound is cost-effective compared to MRI and CT scans, there is scope for developing more advanced MRI or CT versions to detect small lesions in the liver. Similarly, there is a need for an appropriate combination of liquid biomarkers should be used for the investigation of liver carcinogenesis. From a treatment perspective, upon early diagnosis, liver

transplantation is preferred over surgical removal or ablation as it is having less than 15% chance of recurrence[90].

The primary cause of treatment failure in cancer is resistance to available chemotherapy, which advances in relapse. From heterogeneous tumors, cells respond to treatment differently, and a rare small percentage of cells found in quiescent G0 state of the cell cycle can escape the treatment. These cells are inherently resistant to chemotherapy and culprits for relapse. Studies have shown that tumor cells maintain the drug-tolerant state via chromatin-mediated changes after drug treatment[13]. The drug-tolerant persister (DTP) stage is reversible; however, prolonged exposure to chemotherapeutic drugs results in stable drug resistance property[91–93]. DTP cells have non-random differential gene expressions, implicating chromatin-mediated changes leading to hetero-chromatinization of the transposable elements such as LINE1[94]. Recent findings suggest that ablation of the DTP cell population with FDA-approved epi-drugs impedes the development of resistance and relapse[13,94]. Hangauer *et al.* have shown DTP cells dependence on mesenchymal state and GPX4 (lipid hydroperoxide) for survival. Further, inhibition of GPX4 triggers cell death of DTP cells via the ferroptosis pathway, indicating ferroptosis is required for the survival of DTP cells[95]. Thus, targeting inherently resistant residual cells could be helpful to reduce relapse cases. However, more research on the identification and characterization of DTP cells is required to choose the proper drug combination for treatment purposes.

Targeted drug delivery is the critical factor in improving treatment outcomes and reducing the drug's side effects. Currently, researchers are investigating nanoparticle-mediated drug delivery. Apart from this, modified liposomal formulation showed a successful therapeutic response in HCC due to tumor-directed delivery and low drug load in the system[96]. Albumin is also a suitable

drug-carrier molecule. Albumin-tagged drug has more potent effects compared to an alone drug[97]. Other materials such as dendrimers, micelles, polysaccharides, and silica also used as carrier molecules[98–100]. Still, the hunt for an effective delivery system continues for targeted delivery.

Conclusion

Existing diagnostic methods are inadequate for the early detection of HCC. Similarly, implemented treatment modalities are unsuccessful in improving the survival of patients and have cytotoxicity on normal cells. The use of credible biomarkers in the prognosis of HCC is essential to reduce mortalities due to the disease. In years to come, clinicians should focus on patient stratification based on molecular signatures and decide the treatment strategy to achieve maximum therapy outcome. The development of a combinatorial regime consisting of epi-drugs is an urgent need to counter-strike the tumor mass.