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Hepatocellular Carcinoma: Can Circulating Tumor Cells and Radiogenomics Deliver Personalized Care?

Richard L. Hesketh, MBBCh, MPhil¹, Andrew X. Zhu, MD, PhD^{2,4}, and Rahmi Oklu, MD, PhD^{3,4,*}

¹West Middlesex University Hospital, London, United Kingdom

²Massachusetts General Hospital, Division of Hematology/Oncology, Boston, MA

³Massachusetts General Hospital, Division of Vascular Imaging and Interventions, Boston, MA

⁴Harvard Medical School, Boston, MA

Abstract

The 'omics revolution is facilitating a personalized approach to improving outcome by refining diagnosis, staging, treatment and monitoring of hepatocellular carcinoma (HCC). Furthermore, the promise of being able to target a range of specific tumor drivers at a molecular level offers exciting new therapy prospects for a disease that is notoriously difficult to treat. We provide a unique perspective combining our understanding of the molecular mechanisms of HCC development with the potential of circulating tumor cells and radiogenomics to change the drivers of decision-making used in current practice.

Keywords

Hepatocellular carcinoma; radiogenomics; circulating tumor cells

Introduction

Hepatocellular carcinoma (HCC) is the most common primary liver cancer with an estimated 782,000 new cases and 746,000 deaths worldwide in 2012 resulting in it now being the second most common cause of cancer related death.[1]The disease burden remains predominantly in the Far East and Sub-Saharan Africa, while in the United States HCC is the eighth leading cause of cancer death.[2, 3]Importantly, the seven cancers that result in more deaths per year than HCC are all decreasing in incidence, while that of HCC has doubled over the last two decades. HCC is a silent killer displaying minimal symptoms in the early stage of disease and this, combined with the current lack of specific biomarkers, often results in the detection of disease at a stage where current treatments rarely induce remission. Current staging systems take no account of the molecular characteristics of the tumor and are based solely on tumor size, spread, liver function and performance status. For tumors that have not metastasized to distant sites and fulfill strict criteria, transplantation

*Corresponding author For all correspondence, please contact: Rahmi Oklu, MD, PhD, Massachusetts General Hospital, Vascular Imaging and Interventions, 55 Fruit Street, 290 Gray/Bigelow, Boston, MA 02114, Tel: (617) 726-8314, roklu@mgh.harvard.edu.

remains the best treatment as it removes not only the known primary tumor but also any undetected satellite lesions within the liver and treats underlying cirrhosis. However, limited organ availability means that disease progression is common while waiting for a donor. Therefore, tumors detected early are surgically resected with excellent results: the five-year survival rate has been reported to be up to 100% for tumors under two centimeters in diameter. In stark contrast, the five-year survival rate is under 10% for patients with larger tumors. With these statistics in mind, research in two broad categories is likely to have the greatest impact on overall survival: (i) methods to detect tumors earlier enabling surgical / interventional techniques to be applied to a larger cohort of patients and (ii) improvements to treatment modalities when surgical techniques are not applicable (i.e. advanced disease). Summarizing our present understanding of the molecular and etiological mechanisms of the disease, this review will discuss the novel approaches in these two categories that have the potential to refine current staging algorithms, improve prognostics and ultimately guide therapy.

Etiology of HCC

The initial insults that induce carcinogenesis *via* both direct and indirect mechanisms have been well defined for HCC, and are more diverse than for any other cancer. The most prevalent risk factors are liver cirrhosis (of any etiology) and chronic infection with either hepatitis B virus (HBV) and/or hepatitis C virus (HCV). Prevalence of these viruses largely determines the huge regional variation in HCC incidence. The prevalence of HCC is highest in the Far East and Sub-Saharan Africa, where HBV is endemic (China accounts for 55% of HCC cases globally, and 99% of these are secondary to HBV infection). However, vaccination programs in these countries have dramatically reduced both HBV and HCC incidence, a trend that is expected to continue. Underlying the increasing prevalence of HCC in the United States is a rise in HCV infections between the 1960s and 1980s. Although the HCV infection rate is now falling, the latent complications of HCV means that HCC incidence is likely to continue to rise over the next decade.[3]

Etiological agents with direct carcinogenic effects

Clonal immortalization and mutagenesis occurs secondary to the high cellular turnover and inflammation characteristic of cirrhosis. However, HCC also develops rarely in the absence of cirrhosis, and data are starting to emerge that explain direct carcinogenesis. It seems a reasonable assumption that the different etiological drivers of HCC would create a unique molecular signature, however this picture is only partially understood. A notable exception is aflatoxin B₁ (AFB₁), which is exclusively associated with a dose-dependent AGG to AGT transversion at codon 249 of *TP53*. [4] The hepatitis viruses have a variety of oncogenic mechanisms producing a wide range of molecular effects. HBV DNA integration into the host genome is postulated to cause the direct induction of oncogenesis *via* two mechanisms: (i) expression of virally encoded oncoproteins and (ii) alteration of host gene function. Critical among the former is HBx, which activates multiple signalling pathways. As a positive-sense RNA virus lacking reverse transcriptase, HCV is unable to integrate within the host genome. However, several viral core and envelope proteins have been identified that have direct tumor promoting effects.[5] In particular, HCV protein NS5A results in β -

catenin activation and consequently an increase in MYC transcription, a key driver of many tumors including HCC.[6]

Genetic Drivers of HCC

Reflecting the varied etiology, HCC tumors show extreme genetic heterogeneity. Chromosomal instability resulting in somatic copy number variation is a prominent feature of HCC, with recurrent allelic deletion of 1p, 4q, 6q, 8p, 9p (*CDKN2A*), 10q, 13q (*RBI*), 16q and 17p (*TP53*) and amplification of 1q, 6p, 8q (*MYC*), 17q and 20q. Whole genome sequencing (WGS) has revealed between 4,886 and 24,147 somatic point mutations.[7-11] In principle, any of the components of a signaling pathway may undergo mutation, although in practice more frequently susceptible genes emerge from genetic screens. *TP53* and *CTNNB1* (β -catenin) are the most frequently mutated genes and are associated with a poorer prognosis, but the relatively low frequency of individual mutations suggests that hepatocarcinogenesis results from an accumulation of multiple, infrequent mutations and cooperation of two or more aberrantly activated signalling pathways.[12-14] Additionally, in keeping with other tumors, HCC involves numerous epigenetic changes regulating gene expression including deregulated DNA methylation, histone modifications and expression of microRNAs (miRNAs). Numerous studies have reported the prognostic implications of individual gene expression level and epigenetic modifications and more than twenty prognostic molecular signatures have been reported.[15] Improving on previous signatures, a score based on the expression levels of five genes (*HNF1*, *RAN*, *RAMP3*, *KRT19* and *TAF9*) has been validated as an independent predictor of survival across HCC samples of differing etiology.[16] The advent of single molecule sequencing, currently in its infancy, is set to reveal the complete epigenetic picture and further refinement of prognostic signatures will occur.

It seems probable that, in common with practically all-solid tumors, the most potent molecular driver of HCC is the transcription factor MYC. MYC can directly regulate the expression of some 15% of human genes and it has indirect effects on many more through its regulation of inhibitory miRNAs that have direct effects on cell cycle control and chromatin remodelling. The MYC protein rarely undergoes mutation but manifests its oncogenic effects through over-expression that can arise from multiple mechanisms, most commonly gene amplification or aberrant activities of the upstream signalling components of the RAS-MAPK pathway, or more commonly the WNT pathway.

Dysregulation of the WNT pathway leads to the over-expression of β -catenin, activating transcription of numerous genes (including MYC) and is proposed as a key initiator of hepatocarcinogenesis. WNT signalling, along with TGF- β , Notch, and Hedgehog signalling pathways, are implicated as regulators of cancer cell 'stemness'. Cancer stem cells (CSCs), a sub-population of tumor initiating cells capable of indefinite self-renewal and differentiation, are hypothesized to regulate tumor growth, metastasis and recurrence. They are thought to be particularly chemo-resistant and therefore there is great interest in the development of therapeutics that target components of the controlling pathways.[17]

The Future of Diagnosis

Current surveillance methods for HCC are magnetic resonance imaging (MRI) or ultrasonography and alpha-fetoprotein (AFP), with high-risk populations being offered screening every six months. MRI is the gold standard but still only detects 80% of lesions under two centimeters in diameter compared to 60% and 59% detected by computed tomography (CT) and ultrasound, respectively.[18, 19] The sensitivity and specificity of AFP has been reported at 39 – 64% and 76 – 91%, respectively.[20] In addition to the lack of sensitivity of the screening tests, less than 20% of high-risk patients receive the recommended frequency of screening.[21] A screening test is required that offers an improved sensitivity and specificity compared to the current methods but also needs to be quick, minimally invasive and cost effective to maximize screening uptake.

A serum marker with the sensitivity and specificity to replace radiological screening has been elusive. AFP was first identified as a marker for HCC over 40 years ago and, despite its low sensitivity and specificity, remains in use today. Metabolomic and proteomic studies aiming to identify additional early markers of HCC have so far used small subject numbers and been of fragmented design, and many of the seromarkers studied do not significantly improve sensitivity and specificity relative to AFP (Table 1). Detection of known antigens, including AFP, using IgM immunoglobulins and enzyme-linked immunosorbent assay (ELISA) have shown improved sensitivity compared with traditional assays.[22, 23] However, as a screening test ELISAs are impractical: they are expensive, time consuming and require a skilled technician.

Circulating Tumor Cells

The difficulty in identifying suitable serum markers for tumor detection is not limited to HCC. No cancer has a satisfactory serum marker. Therefore, rather than attempt to detect the molecules released by a tumor into the circulation, one proposed alternative is to try and detect the tumor directly. The presence of tumor cells in the systemic circulation is a prerequisite for metastasis, a concept that has prevailed for over a century. Traditionally, metastasis has been considered as a late phenomenon, however recent data suggest that metastasis can occur at an early stage of tumor development but is a highly inefficient process.[24] It is estimated that approximately 10^6 cells per gram of tumor are shed into the circulation each day, which would suggest that CTCs are detectable well before the tumor is visible using imaging.[25] The short half-life results in the presence of approximately one circulating tumor cell (CTC) in one billion blood cells being present in the circulation at a given time. This minute quantity of CTCs presents a huge challenge to both the sensitivity and specificity of detection assays. Numerous techniques have been developed for this purpose, based either on the physical (size, density or charge) or biological (cell surface expression, tumor specific RNA or cell free DNA) properties of CTCs. Contamination of enriched CTCs with leukocytes when physical properties are utilized as the separation strategy, has led to specific antibody-based enrichment techniques often being the preferred method.

Certain solid tumors, particularly lung and breast, have been the subject of intensive CTC research over the last few years. Investigation in HCC has lagged behind, partly because of

its perceived lack of importance and also due to unique technical difficulties. Epithelial cell adhesion molecule (EpCAM) antibody-coated magnetic beads are widely used for CTC detection (CellSearch® system, Janssen Diagnostics, Raritan, NJ). Unlike most epithelial tumors, EpCAM is not expressed in the majority of HCCs and nor are there established complimentary antibodies to any other HCC-specific surface markers.[26] Furthermore, it is thought that a crucial step in the development of metastasis is the epithelial-mesenchymal transition (EMT), a process that describes the steps required for tumor cells to acquire an invasive phenotype. During this transition, many epithelial markers are lost, including EpCAM, severely limiting the usefulness of EpCAM based detection methods.[27]

In HCC, several reports of novel approaches to the detection of CTCs have emerged. Using multicolor flow cytometry to identify CD45⁻CD90⁺CD44⁺ circulating stem cells (a subset of CTCs) in samples taken pre-hepatectomy, Fan et al. correlated levels >0.01% with increased recurrence and decreased survival.[28] In another study using antibody-coated magnetic beads targeting the asialoglycoprotein receptor (ASGPR) exclusively expressed on the surface of hepatocytes, Xu et al. demonstrated 81% and 100% sensitivity and specificity, respectively, for HCC CTCs.[29]

Using antibody-based techniques, CTCs can be isolated either by positive selection, whereby the antibody target is on the surface of the CTC, or by negative selection to deplete a sample of the other cells present, leaving the CTCs. The latter avoids the problem of variable receptor expression on CTCs. The CTC-iChip is a microfluidic device using a three-step process to isolate CTCs and can be configured in a negative or positive selection mode. In a negative selection mode, leukocytes are tagged with immunomagnetic beads targeting CD45 and CD15. Inertial focussing then segregates nucleated cells from red blood cells. In the final step, magnetic-activated cell sorting (MACS) step separates CD45⁻CD15⁻ CTCs from the tagged leukocytes. Utilizing the negative selection method, the CTC-iChip has demonstrated a CTC recovery rate of 97% and a sorting rate of up to 10⁷ cells per second.[30]

If the sensitivity of assays can be perfected, CTCs could enhance every stage of cancer management from screening to therapy monitoring. CTC detection represents a highly specific tool for cancer screening which would identify high risk patients that need regular imaging. Enumeration of CTCs has been shown to be an independent prognostic marker in many tumors which could lead to the refinement of current staging algorithms. In addition to simple enumeration, microfluidic techniques that capture viable CTCs in suspension permit further analysis by immunohistochemical, genomic or transcriptomic techniques. This presents the possibility of using CTCs as a surrogate for biopsy of the primary tumor – the so-called “liquid biopsy.” This could render invasive tissue biopsy superfluous and, as a minimally invasive peripheral blood test, the liquid biopsy could be repeated at regular intervals during treatment facilitating response monitoring and potentially allowing therapy to be tailored to genotypic and phenotypic change.

Radiogenomics

Radiology is already an integral part of the management of the vast majority of conditions on account of its ability to provide anatomical and morphological details. The increasing

detail available from modern CT and MRI has revealed that tumors, previously considered as a single entity, have individual imaging characteristics, which are generally poorly understood and consequently often ignored. The recognition of these traits may provide vital additional information enabling more accurate prediction of tumor behaviour including response to therapeutic interventions and prognosis. It has been hypothesized that tumor imaging phenotypes correlate with the underlying genotype, and subsequently radiology can be used as a surrogate for genomics and transcriptomics.[31] Isolated case studies have shown the potential of whole genome sequencing to guide treatment and ultimately improve prognosis.[32] Using radiological phenotype as a surrogate for genomics and transcriptomics, termed radiogenomics, may provide a rapid and cost effective alternative to the introduction of sequencing into routine clinical management.

Radiogenomics is a field very much in its infancy, but initial results in HCC have been promising. On the basis of just 28 different imaging traits, the expression of 74% of 6,732 genes could be predicted. Additionally, tumors with internal arteries and an absence of hypodense halos were associated with increased expression of 91 genes resulting in a 12-fold increased risk of microscopic venous invasion. The presence of internal arteries was also an independent marker for a poor prognosis.[33] In addition to having potential prognostic implications, radiogenomics may be able to directly guide therapeutic decisions. A 61 gene signature, predictive of doxorubicin resistance in various human tumor cell lines, was applied to HCC tumors and six imaging traits were examined. Of the six imaging traits, a poorly defined tumor margin was predictive of the doxorubicin resistance signature.[34]

Further advances in radiogenomics may be possible with metabolic imaging. Although fluorine-18 fluorodeoxyglucose (FDG) positron emission tomography (PET) currently lacks sensitivity for tumors under 5 cm and therefore plays no role in diagnostic imaging, high uptake of FDG correlates with a high-grade of HCC.[35, 36] Another development is the use MRI to monitor tumor activity using hyperpolarized ^{13}C labelling of various metabolites. The inhibition of flux of ^{13}C label from pyruvate and lactate is measurable within 24 hours of chemotherapy and is a proposed method to give an early indication of tumor response. [37] Expansion of the pool of labelled metabolites will provide further insight into cellular activity and offers a further tool with which to develop correlations between imaging phenotypes and the tumor genotype.

Implications for Future Therapy

In addition to the increasing availability of new methods of diagnostic and staging techniques, treatment modalities are also being refined. The main aim of improved screening tests is to increase the proportion of patients diagnosed with localized disease. This will put increased demand on surgical services including the already stretched transplant service. The increasing gap between supply and demand for liver transplantation is being addressed in a number of ways, including the prospect of bioartificial organ transplantation. More effective methods of percutaneous ablation are also being developed to improve homogenous tissue death and reduction of side effects.[38]

Advanced HCC was, until recently, the only solid tumor without a survival-prolonging systemic chemotherapy. The introduction of sorafenib, a multi-kinase inhibitor of RAF, vascular endothelial growth factor receptor (VEGFR) and platelet-derived growth factor receptor- β (PDGFRB) has increased life expectancy by 35%. However, the dire prognosis means this equates to just over two months.[39] The genomic revolution has revealed a multitude of other potential therapeutic targets in HCC. The concept of personalized therapy utilizes a battery of agents to target selectively the driving mutations identified by molecular characterization of the individual tumor. However, the current lack of drugs specific for many key components of signalling pathways and the ability of tumors to develop resistance to targeted therapy has limited success thus far. Furthermore, with the notable exception of β -catenin, the majority of mutations observed in HCC result in a loss of function. Gene therapy (usually by adenoviral delivery), DNA vaccines, oncolytic viruses and small interfering RNAs are just a few of the newer methods potentially capable of targeting tumors driven by loss of function mutations.[40] In addition to a lack of targeted drug therapies, a number of other key factors limit effective treatment. The unique tumor microenvironment consisting of irregular blood flow, acidity, high interstitial pressure, hypoxia and an abnormal extracellular matrix composition is a major barrier to intracellular drug delivery. Through their ability to “normalize” the tumor microenvironment and improve drug delivery, a combination of anti-angiogenic drugs and ablation techniques with chemotherapy may improve overall efficacy.[41]

The alternative approach to personalized therapy is to target a signalling component that is absolutely essential for tumor growth. So far, this approach has been unsuccessful due to the lack of a target that is vital for tumor survival and the incredible ability of a tumor to rapidly evolve and develop mechanisms of drug resistance. It is the latter that has particularly hindered all molecular targeted therapy to date and is particularly well documented in the cases of imatinib, vemurafenib and erlotinib/gefitinib.[42] Lying at the crossroads of multiple molecular pathways, MYC is theoretically the most attractive target that has been identified for a single target molecular therapy for cancer. Systemic MYC inhibition has been shown not only to arrest growth, but to induce complete regression of advanced KRAS-driven lung adenocarcinomas in mice. Furthermore, to the surprise of many, the inhibition of MYC did not induce widespread toxicity and side effects. The next step of this work is to devise a method of MYC inhibition applicable to the clinical setting.[43]

Conclusions

The occult nature of HCC makes minimally invasive diagnostic tests of paramount importance and the most likely way of improving survival is a screening test that leads to the diagnosis of tumors at a stage when surgical treatment remains a curative option. With the knowledge that CTCs may be present (albeit in minute quantities) before a tumor is even visible by current imaging modalities, they have the potential to be the sensitive and specific blood screening test that has so far been elusive in HCC. As well as improved sensitivity compared to today's methods of screening, as a peripheral blood test it could be introduced to the clinic with minimal expense and improve the proportion of high-risk patients receiving screening.

To manage a disease that is as heterogeneous as HCC a treatment regimen tailored to the underlying molecular characteristics seems a sensible approach. The development of 'omic technologies has contributed hugely to the understanding of oncogenic mechanisms over the last decade and led to the identification of numerous therapeutic targets. However, the abundance of new research data has so far produced only modest benefits for clinicians managing the disease. This has been partly due to the cost of implementation but also due to the difficulty of clinical correlations from the vast data outputs of genome wide studies. The implications of incorporating genomic data into staging and treatment algorithms are considerable improvements to predicting prognosis, guiding treatment options and monitoring response to therapy. To obtain molecular data about a tumor in a rapid and cost-effective manner, CTCs and radiogenomic analysis are particularly attractive modalities as they can be implemented without drastic changes to the clinic setup (Fig. 1). Their addition to the clinicians' toolkit would facilitate the new and exciting prospect of managing HCC with personalized treatment targeting the specific molecular aberrations of the individual tumor.

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Fig. 1. A proposed algorithm for incorporating CTCs and radiogenomics into screening, diagnosis and management of HCC

The inset shows the potential applications of CTCs and the principle of radiogenomics: creating an association map between imaging traits and microarray data to allow prediction of gene expression based solely on the imaging traits. IHC – immunohistochemistry. aCGH – array comparative genomic hybridization.

Table 1

Current and future seromarkers.[20,22,33]

Serum factor	Sensitivity (%)	Specificity (%)	Comment
AFP	39 – 64	76 – 91	Also elevated in pregnancy, active hepatitis, cirrhosis, liver metastases, hepatoblastoma and other neoplasms (primarily tumors of the digestive tract, yolk sac and germ cell tumors). Concentration correlates with tumor size.
AFP-L3	45 – 90	>95	Major isoform of AFP raised in HCC. Sensitivity dependent on lesion size. AFP-L3/AFP ratio >10% indicates poorer prognosis.
α-L fucosidase	80 – 90	70 – 98	Lysozymal enzyme. Elevated in diabetes, pancreatitis and hypothyroidism. Also has potential for monitoring response to therapy.
Des-γ-carboxy prothrombin *	48 – 62	81 – 98	An abnormal prothrombin protein produced as a result of an acquired defect in post-translational carboxylation. Poor in detecting small tumors. Sensitivity and specificity increase to 74% and 87%, respectively, when combined with AFP.
Glypican-3	50	100	Heparan sulphate proteoglycan that acts as a tumor suppressor. Modulates cell proliferation by inhibiting fibroblast growth factor 2 (FGF2) and bone morphogenetic protein 7 (BMP7) activity.
Golgi protein-73	69	75	Resident Golgi glycoprotein with increased expression in diseased hepatocytes, particularly early HCC cells.
Squamous cell carcinoma antigens (SCCAs)	84	46	Members of the ovalbumin family of serine proteinase inhibitors. Also increased in other epithelial malignancies. Inversely correlated with tumor size. SCCA IgM IC has 100% and 70% sensitivity and specificity, respectively.

* Also known as protein induced by vitamin K absence (PIVKA)