Role of Molecular Biomarkers in Liver Transplantation for Hepatocellular Carcinoma

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Patient selection and organ allocation for liver transplantation (LT) in patients with hepatocellular carcinoma (HCC) relies predominantly on clinical parameters, such as tumor burden (ie, radiological imaging). Patients transplanted within Milan criteria have outstanding outcomes with a 5- and 10-year survival of 70% and 55%, respectively. Tumor recurrence after transplantation is rare in these patients (10%); however, treatment options upon recurrence are generally limited, and outcomes are poor. There are also several studies showing how a subgroup of patients with tumors outside the Milan criteria might achieve comparable outcomes to patients within Milan criteria. In other words, the size and number of tumor nodules does not always reflect tumor biology, which could be better captured using molecular proxies for cancer aggressiveness. Over the last decade, we have significantly improved our understanding of the molecular landscape of early stage HCC. This includes the development of molecular classification, identification of prognostic and mutational signatures, and potential mechanisms of hepatocarcinogenesis. Some molecular markers have already proven useful to predict tumor-related outcomes in HCC patients after LT. Most of these analyses are limited to tissue-derived biomarkers, which limits their implementation in clinical practice because tissue biopsy is not required for HCC diagnosis. Minimally invasive alternative tools, such as liquid biopsy, are being increasingly explored and could help to individualize risk stratification for patients with HCC who will benefit from LT despite being outside the accepted clinical criteria.

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It is estimated that in 2030, more than 1 million people will die due to liver cancer worldwide.1 In the United States, the liver cancer death rate increased 43% between 2000 and 2016.2 With a 5-year survival of 18%, it is the second most lethal malignancy after pancreatic cancer.3 Hepatocellular carcinoma (HCC), the most common form of primary liver cancer, typically occurs in patients with chronic liver disease due to hepatitis B virus (HBV) and hepatitis C virus (HCV) infections, alcohol-use disorder, and nonalcoholic fatty liver disease in the context of diabetes and metabolic syndrome.4

According to guidelines,5,6 tumor staging of HCC is based on the Barcelona Clinic for Liver Cancer (BCLC) classification algorithm, which also provides treatment recommendations based on tumor burden, degree of liver dysfunction, and clinical performance status.4 Curative treatment options are limited to early stage HCC (BCLC stages 0 and A) and include liver resection, ablation, and liver transplantation (LT). Initial results of LT for HCC without any specific listing criteria were dismal,7,8 with aggressive tumor

Abbreviations: AFP, alpha-fetoprotein; BCLC, Barcelona Clinic for Liver Cancer; CK19, cytokeratin 19; CTC, circulating tumor cell; FAL, fractional allelic imbalance; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; LDLT, living donor liver transplantation; LT, liver transplantation; MELD, model for end-stage liver disease; miR, microRNA; PD-1, programmed cell death 1; PD-L1, programmed cell death ligand 1; STORM, Sorafenib as Adjuvant Treatment in the Prevention of Recurrence of Hepatocellular Carcinoma.

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recurrence early after transplantation. It was not until 1996 when the landmark article from Mazzaferro et al. demonstrated that LT was a promising option for patients with a limited tumor burden and without macrovascular invasion or extrahepatic spread. These so-called Milan criteria included patients with a single nodule <5 cm or up to 3 nodules with none larger than 3 cm. Survival for patients transplanted within this criteria is approximately 70% and 55% at 5 and 10 years, respectively. There have been different attempts to expand these criteria, which generally result in worse outcomes. The scarcity of donors has also become an increasing problem, leading to increased waiting times and the possibility of tumors progressing beyond a point where transplantation may no longer be an option. Treatment on the waiting list helps decrease dropout.

Tumor downstaging, generally using transarterial therapies, has shown efficacy and is currently accepted by the United Network for Organ Sharing as an option in patients exceeding conventional criteria. All these data underscore the need for new methods to better quantify tumor aggressiveness and allocate organs accordingly.

In the last 4 years, there have been major advancements in the treatment of patients at an advanced stage of HCC (BCLC C). Sorafenib was the only effective agent for about 10 years, but now there are numerous systemic therapies that have shown efficacy in phase 3 clinical trials. These drugs include lenvatinib in first-line treatment and regorafenib, cabozantinib, and ramucirumab (for patients with alpha-fetoprotein [AFP] >400 ng/mL) in second-line treatment.

In addition to tyrosine kinase inhibitors, immune checkpoint inhibitors, such as nivolumab and pembrolizumab, which are monoclonal antibodies against programmed cell death ligand 1 (PD-L1), have shown promising tumor response rates nearing 20% in phase 2 clinical trials, which has led to their approval by the US Food and Drug Administration under the accelerated program. These promising results were not confirmed in large phase 3 trials. A recent combination of the monoclonal antibodies atezolizumab (against programmed cell death 1 [PD-1]) and bevacizumab (against vascular endothelial growth factor) was able to significantly improve overall survival and progression-free survival in a phase 3 clinical trial against sorafenib in first-line treatment, likely establishing a new standard for first-line treatment. There are limited data on the role of these therapies in the transplant setting, either to treat recurrence or to delay progression in patients on the waiting list. Also, it is unclear if molecular predictors of response to these therapies could help identify patients suitable for effective downstaging and subsequent LT. This review will summarize the current knowledge on molecular biomarkers in LT for HCC and their prospects for future clinical implementation.

Molecular Classification of HCC

The introduction of next-generation sequencing technologies has allowed a comprehensive analysis of the molecular landscape of HCC, mostly using resection specimens from patients at an early stage. The most commonly mutated genes are TERT promoter (40%-60%, telomerase maintenance, and gain of function), TP53 (~30%-35%, cell cycle control, DNA repair, and loss of function), and CTNNB1 (~30%-35%, beta-catenin/Wnt signaling, and gain of function). Other less frequent mutations affect ARID1A (chromatin remodeling), AXIN1 (Wnt signaling), RPS6KCA (phosphoinositide 3-kinase/mammalian target of rapamycin signaling), and RB1 (cell cycle control). The underlying etiology of the liver disease seems to affect the frequency of altered genes. For example, CTNNB1 mutations are more common in patients with alcohol-related liver disease, whereas HBV-related HCCs are more frequently mutated in TP53.

Molecular alterations in HCC are not restricted to point mutations, and they can also include DNA amplifications, gene expression deregulation, and
aberrant DNA methylation.\textsuperscript{(30)} Integration of all these layers of molecular information is the basis for the development of molecular classifications of HCC. These allow practitioners to group patients in the same clinical stage but with different molecular alterations. Broadly speaking, HCC can be classified into 2 groups: the proliferation subclass or the non-proliferation subclass.\textsuperscript{(31,32)} The proliferation subclass is associated with HBV etiology, progenitor-like features, \textit{RAS/MET/AKT} pathway activation, higher serum AFP levels, and worse clinical outcomes.\textsuperscript{(33)} The non-proliferation subclass is associated with HCV etiology, hepatocyte features, classical Wnt/beta-catenin signaling, and a better outcome.\textsuperscript{(33)} There are also reports defining the key molecular immune features of HCC, including a recent immune-based molecular classification of HCC.\textsuperscript{(34)} The immune subclass, described in ~30\% of patients, is characterized by high expression levels of PD-L1, PD-1, and markers of T-cell cytolitic activity.\textsuperscript{(34)} Also, numerous prognostic gene signatures have been reported in HCC.\textsuperscript{(24,30,33)} These are sets of genes whose expression tightly correlates with clinical outcomes; some of them are even tested in LT as discussed below. The most frequent DNA copy number variations in HCC affect \textit{MYC, CCND1, VEGF, FGF19, and TERT} (all high-level focal amplifications), and \textit{CDKN2A, RB1, AXIN1, and IRF2} (all homozygous deletions).\textsuperscript{(27,28,33)} Furthermore, key aberrations in DNA methylation between HCC and nontumoral tissue have been found in \textit{CDKL2, FOXE3, and SEPT9}.\textsuperscript{(35)}

**Clinical Selection Criteria for Patients With HCC**

Shortly after Model for End-Stage Liver Disease (MELD) scoring was adopted for organ allocation by the United Network for Organ Sharing, patients with HCC within the Milan criteria were granted exception points because their biological MELD scores would not reflect their high risk of wait-list dropout due to tumor progression and tumor-related death.\textsuperscript{(36)} Subsequent analyses confirmed a disproportional advantage for patients with HCC over patients with end-stage liver disease without HCC, which resulted in several modifications of this system over the years.\textsuperscript{(36)} Despite this, the proportion of LT due to HCC has steadily increased.\textsuperscript{(37)} It remains a common indication for LT both in terms of wait-list registrants and recipients (24\% and 27\% in 2015, respectively).\textsuperscript{(38)} Considering the discrepancy between the number of recipients with HCC and the number of available organs and our lack of tools to accurately monitor tumor aggressiveness, it is imperative to develop new methods and better match patients with organs on an individual basis. This personalized listing policy could exclude patients with very aggressive tumors who would not benefit from LT due to either rapid progression or high recurrence rates as a result of early tumor dissemination. On the other hand, those patients having tumors with favorable molecular indicators, including low dissemination rate, limited invasion capacity, and cell growth, could be rescued as good candidates for LT regardless of tumor burden.

Several staging systems have been proposed to identify patients with favorable outcomes, despite their tumors exceeding the Milan criteria (eg, University of California, San Francisco criteria, Up-to-7 criteria, Dallas criteria, Asian criteria, Kyoto criteria, and Toronto criteria\textsuperscript{(39)}). AFP has also been used to better risk stratify patients in the transplant setting. AFP is a well-known prognostic biomarker in HCC\textsuperscript{(5,6)} and it was recently shown to also predict response to ramucirumab.\textsuperscript{(17)} The incorporation of AFP levels with tumor burden (ie, Milan criteria) was found to improve patient selection, although it yielded only a moderate accuracy of 72\% to predict 5-year posttransplant survival.\textsuperscript{(11)} The main problem with AFP is that not all aggressive tumors express this marker and that the percentage of patients expressing it increases with tumor stage. Thus, it seems that these clinical parameters are suboptimal for patient selection. In theory, molecular biomarkers of tumor biology, ideally ones that are minimally invasive, could help implement personalized selection criteria, rationalize organ use, and maximize positive clinical outcomes (Fig. 1).

**Molecular Biomarkers for HCC Risk Stratification in LT**

Numerous studies have investigated the utility of molecular biomarkers to predict outcomes in patients following LT for HCC (Table 1). A small study defined a molecular subclass of aggressive HCC tumors based on the presence of TP53 mutations, high fractional allelic loss, significant hypomethylation of 8 tumor suppressor genes, and the absence of CTNNB1 mutations.\textsuperscript{(40)} However, the prognostic role of TP53...
mutations in HCC is still unclear. These features, which are similar to those of the proliferation class reported earlier,\(^{33}\) were predictive of metastatic recurrence and shorter recurrence-free survival in 25 HCC patients who underwent LT.\(^{40}\) Along with DNA-based markers, gene expression signatures have been associated with worse outcomes after LT in patients with HCC outside Milan criteria.\(^{41}\) Briefly, the authors performed whole transcriptomic profiling in a large cohort of 132 patients transplanted due to HCC outside Milan criteria. They identified the S2 molecular subclass\(^ {32}\) and progenitor cell markers (ie, CK19 signature\(^ {31}\)) as independent predictors of overall survival and recurrence after LT, respectively.\(^ {41}\) Likewise, in multinodular HCC, tumor evolutionary distance, which is a proxy for molecular dedifferentiation that is measured from genome-wide single-nucleotide polymorphism data between the cirrhotic liver and tumor nodules and between the tumor nodules themselves, is correlated with higher rates of recurrence and worse recurrence-free survival.\(^ {42}\) Despite a small sample size, authors validated their findings in an independent cohort.\(^ {42}\) In a study of 183 patients who received LT due to HCC, including patients before the implementation of Milan criteria, the fractional allelic imbalance (FAI) rate index was used to compare the acquired mutational load between different tumors.\(^ {43}\) The FAI was determined from the microdissected tissue site displaying the greatest amount of acquired allelic loss, and it was the strongest predictor of tumor recurrence followed by other clinicopathological parameters, such as vascular invasion, tumor number, or hepatic lobar involvement.\(^ {43}\) The authors built an integrated prognostic model combining FAI and vascular invasion (ie, the Pittsburgh criteria) able to rescue an additional 39% of patients for LT with a 5-year tumor-free survival rate of 88%. These patients would have been otherwise excluded as per Milan criteria.\(^ {43}\) Recently, allelic imbalance was evaluated in more detail in 71 patients who received LT for HCC, 18 of whom developed a tumor recurrence.\(^ {44}\) The authors evaluated 19 microsatellites and reported 3 loci (D3S2303, D9S251, and D9S254) as predictive of HCC recurrence after LT, including early recurrence.\(^ {44}\) These data suggest that, in patients outside of Milan criteria, FAI could identify those at high risk of tumor recurrence after LT.\(^ {45}\)

Besides DNA changes and aberrant gene expression, microRNAs (miRNAs, miR) have extensively been evaluated as prognostic biomarkers in HCC.\(^ {46,47}\) In LT, several reports have identified candidate miRNAs as predictors of tumor recurrence. In a study including 70 patients who received LT for HCC, increased tumoral expression of miR-18a and decreased expression of miR-199a-5p were correlated with clinical features of tumor aggressiveness (ie, high levels of tumor markers and portal venous invasion) and a high recurrence rate.\(^ {48}\) These markers regulate the expression of tumor necrosis factor α–induced protein 3 (miR-18a),

| DNA Alterations          | High Risk                           | Low Risk                   |
|--------------------------|-------------------------------------|----------------------------|
| TP53 mutation            | CTNNB1 mutation                     |
| Fractional allelic loss  |                                     |
| Hypomethylation of tumor suppressors |                     |
| Transcriptomics          |                                     |
| S2 class                 |                                     |
| Progenitor cell features (CK19 signature) |                     |
| miR                      |                                     |
| miR-18a                  |                                     |
| Exosomal miR-92b*        | miR-199a-5p                         |
| Exosomal miR-718*        |                                     |
| Histological Parameters  |                                     |
| Vascular invasion        |                                     |
| Clinical Parameters      |                                     |
| AFP levels               |                                     |
| Tumor burden             |                                     |
| References | Number of Patients | Milan Criteria | Molecular Marker | Source of Biomarker | Outcome Prediction |
|------------|--------------------|----------------|------------------|---------------------|--------------------|
| Nishida et al. (40) (2018) | 25 | | TP53 mutations, high fractional allelic loss, hypomethylation of 8 tumor suppressors, absence of CTNNB1 mutations | Tissue | Metastatic recurrence, shorter recurrence-free survival |
| Miltiadous et al. (41) (2015) | 132 | Outside | S2 molecular subclass and progenitor cell markers (ie, cytokeratin [CK19] signature) | Tissue | Recurrence and worse overall survival |
| Heits et al. (42) (2018) | 25 | Multinodular HCC (≥2 nodules) | Tumor evolutionary distance* | Tissue | Recurrence and worse recurrence-free survival |
| Dvorchik et al. (43) (2008) | 183 | Inside and outside | Fractional allelic imbalance (FAI) | Tissue | Recurrence |
| Pagano et al. (44) (2019) | 71 | | Allelic imbalance (loci D3S2303, D9S251, and D9S254) | Tissue | Recurrence |
| Morita et al. (45) (2016) | 70 | | Tumoral expression of miR-18a and decreased expression of miR-199a-5p | Tissue | Recurrence |
| Liese et al. (46) (2016) | 40 | Inside and outside | Tumoral expression of miR-214 and miR-3187 | Tissue | Recurrence |
| Barry et al. (47) (2012) | 64 | Inside and outside | Panel of 67 miRs | Tissue | Recurrence |
| Xie et al. (48) (2016) | 89 | Inside and outside | Panel of 5 miRs (decrease in tumoral expression of miR-122, miR-126, miR-15a, miR-22, and miR-30a) | Tissue | Recurrence |
| Sugimachi et al. (49) (2015) | 65 | | Low expression levels of miR-718 in serum exosomes | Serum exosomes | Recurrence |
| Nakano et al. (50) (2019) | 121 | | High expression levels of miR-92b in serum exosomes | Serum exosomes | Recurrence |

*Measured from genome-wide single-nucleotide polymorphism data among tumor nodules and in contrast to livers with cirrhosis. Abbreviations: CK19, cytokeratin 19; FAI, fractional allelic imbalance.
hypoxia-inducible factor 1 alpha, vascular endothelial growth factor A, insulin-like growth factor 1 receptor, and insulin-like growth factor 2 (all miR-199a-5p).\(^{(48)}\) Another study found expression of miR-214 and miR-3187 correlated with the recurrence of HCC after LT.\(^{(49)}\) The authors developed a prognostic score incorporating miR-214, miR-3187, and Milan criteria, which improved the accuracy to predict HCC recurrence compared with Milan criteria alone.\(^{(49)}\) There are several other examples that report miRNAs as predictors of posttransplant recurrence.\(^{(50,51)}\)

None of these molecular biomarkers are incorporated in clinical practice or recommended in guidelines thus far. The main reason is the need for prospective well-powered studies to definitively establish their ability to accurately predict outcomes in these patients. A companion study investigating a representative subcohort of the Sorafenib as Adjuvant Treatment in the Prevention of Recurrence of Hepatocellular Carcinoma (STORM) trial, which tested the effect of neoadjuvant treatment of sorafenib versus placebo to reduce recurrence rates after liver resection or transplantation, identified activated extracellular signal-regulated kinase signaling and microvascular invasion to be prognostic of poor recurrence-free survival, regardless of sorafenib treatment.\(^{(52)}\) These markers likely represent more proliferative and aggressive tumors, which seem to be the most critical characteristics in the transplant setting as well. Another limitation for their clinical implementation is the need for a tissue biopsy to conduct these analyses. Liquid biopsy has emerged as a minimally invasive alternative approach to analyze tumor components without the need of a tissue biopsy. This concept entails the analysis of DNA, RNA in extracellular vesicles (such as exosomes), or circulating tumor cells (CTCs) that are released by the tumor to body fluids, mostly the bloodstream.\(^{(53,54)}\) Despite the potential impact of liquid biopsy for candidate selection and monitoring, there are a few studies applying this technology in this setting.

Low expression levels of miR-718 in exosomes isolated from the serum of patients receiving living donor liver transplantation (LDLT) was associated with worse overall survival and higher rates of tumor recurrence.\(^{(55)}\) Despite miR-718 being negatively correlated with tumor size and vascular invasion, which are known predictors of worse outcome, it remained an independent predictor for survival after multivariate regression.\(^{(55)}\) Another study first identified miR-92b as a key player for HCC development in a rat model.\(^{(56)}\) Secreted by HCC cells and transferred to natural killer cells, it led to down-regulation of CD69 and natural killer cell–mediated cytotoxicity.\(^{(56)}\) The authors showed how patients with high exosomal levels of miR-92b before and at 1 month after LDLT had higher rates of tumor recurrence.\(^{(56)}\)

The analysis of CTCs as a predictor of HCC recurrence is an appealing method to predict recurrence after LT as the main mechanism of extrahepatic HCC recurrence relates to early dissemination and the metastatic spread of CTCs. In fact, detection of CTCs has been associated with more advanced disease\(^{(57)}\) and early recurrence after liver resection.\(^{(58,59)}\) However, data in the transplant setting remain controversial with 1 study showing shorter recurrence-free survival after LT in patients with detectable CTCs compared with those without CTCs (6 versus 15 months).\(^{(60)}\) Another study showed no definitive predictive capacity from CTCs.

**FIG. 2.** Conceptual framework on how a liquid biopsy could improve molecular-based risk stratification for patients with HCC who are evaluated for LT. (Printed with permission from © Mount Sinai Health System.)
enumeration. The main limitation of these technologies is the relatively low sensitivity and lack of reproducibility across different technologies. The use of single-cell RNA sequencing technologies has been tested in HCC to further define the molecular features correlated with the metastatic capacity of CTCs, but additional data in the LT setting are still needed.

**Future Directions**

Most efforts aiming at improving risk stratification for LT candidates with HCC are focused on extending Milan criteria using tumor number and size and AFP levels. With the predicted increase of organs available as a result of having a cure for HCV, both in terms of decreasing the number of patients requiring a LT and the availability of HCV-positive liver donors, the selection criteria for HCC are likely to change in the short term. This will expand the criteria and facilitate the integration of molecular surrogates of tumor aggressiveness. If developed in the context of liquid biopsy, these markers will overcome the need for a tissue biopsy and allow for a real-time molecular analysis of HCC, which will be crucial to monitor tumor clonal composition and detect aggressive clones while the patient is still on the waiting list (Fig. 2). Blood-based approaches will also conquer internodular and intranodular molecular heterogeneity, which limits the feasibility of single biopsy-based approaches. In addition, molecular biomarkers of response to systemic therapies could help identify good responders and enable medical downstaging. In essence, and as in other areas of precision oncology, there are numerous potential clinical applications of molecular markers in transplant oncology.

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