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REVIEW

Selecting patients with hepatocellular carcinoma for liver transplantation: incorporating tumor biology criteria

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Abstract: Liver transplantation (LT) is the optimal therapeutic option for patients with liver cirrhosis and hepatocellular carcinoma (HCC). Due to universal donor shortage, only the patients with limited tumor burden (under the so-called Milan criteria) are considered as potential candidates for LT in most institutions. It is expected that in the near future, more liver grafts will be available for patients with HCC due to the implementation of new direct antivirals against hepatitis C, leaving a prone scenario to consider expanding Milan criteria. A moderate expansion of Milan criteria could be implemented without increasing the risk of tumor recurrence if patients with favorable biological behavior are carefully selected. Incorporating information regarding tumor biology in the decision-making algorithm would result in a more rational use of LT in patients with HCC. In the present review, surrogate markers of tumor biology are critically evaluated as potential tools to be combined with existing radiological criteria. In addition, the current state of liquid biopsy is discussed, as this cutting-edge technology may reshape the management of HCC in the upcoming years.

Keywords: cell-free DNA, locoregional ablation, alpha-fetoprotein, circulating tumor cells, liquid biopsy, biomarkers

Introduction

Liver transplantation (LT) is the only therapeutic option that is able to cure both hepatocellular carcinoma (HCC) and the underlying liver cirrhosis[1] with 5-year survival rates above 70% in most series, which are superior to any other treatment modality.[2] However, tumor recurrence may affect up to 15% of patients, and should this complication occur, patients have limited therapeutic options and prognosis may be poor.[1] In a setting characterized by donor paucity, it is central to optimize the access to LT. Only the patients with a reduced risk of posttransplant tumor recurrence are granted as potential candidates for LT. The current eligibility criteria for LT are mainly based on tumor burden assessed by dynamic imaging techniques. Most transplant institutions have implemented the so-called Milan criteria:[3] a single nodule with a total diameter ≤5 cm, or up to three nodules with a maximum diameter of ≤3 cm each, in the absence of macrovascular invasion or extrahepatic spreading.

Growing evidence supports a moderate expansion of Milan criteria. The upcoming of the new direct antiviral agents against hepatitis C has dramatically decreased the number of patients accessing the waiting lists for LT due to decompensated cirrhosis,[2,4] thus leaving more donors available to consider expanding Milan criteria or at least to acknowledge downstaging as a routine approach. Previously proposed strategies

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consisted in increasing the number and/or diameter of HCC nodules accepted, being the most popular up-to-seven criteria,[3] the Clínica Universitaria de Navarra (CUN) criteria[5] and the University of Southern California-San Francisco (UCSF) criteria.[6] However, expanding Milan criteria is accompanied by a parallel increase of tumor recurrence rates, leading to the *Metroticket principle*: the further the ride (in terms of pretransplant tumor burden), the highest the price (in terms of tumor recurrence rates).[3]

The path toward a more liberal indication of LT in HCC may be walked with leaden feet. A deeper understanding on HCC biology is still needed, and it is central to implement surrogate markers of tumor aggressiveness in clinical practice, in order to ensure that selected patients will remain HCC recurrence-free after LT.[7] In the present review, surrogate markers of tumor biological aggressiveness are critically evaluated as potential tools to select candidates for LT, either alone or in combination with existing radiological criteria. In addition, the potential role of liquid biopsy in this clinical scenario is analyzed to delineate future directions.

Serum soluble markers

Tumor burden, as assessed by radiological techniques, is a poor surrogate of HCC biological aggressiveness: large or multiple HCCs are not always associated with aggressive biological behavior and conversely some patients with small uninodular lesions may show histological features indicating an evolved invasive phenotype. Serum proteins produced by the tumor may provide additional information about its biological behavior (Table 1). The combination of these biomarkers with radiological assessment may result in a more rational approach to select candidates for LT.[8]

Alpha-fetoprotein (AFP)

AFP is a glycoprotein produced by the liver during fetal life and by a variety of tumors including HCC.[9] Serum AFP levels are widely available in clinical practice and they have been traditionally considered a surrogate of tumor burden.[10] However, AFP is over-expressed in only 60%–70% of patients with HCC, while false positives may occur in acute and chronic hepatitis from different etiologies.[11] As a conse-

Table 1 Summary of serum biomarkers with prognostic capacity in patients with hepatocellular carcinoma awaiting liver transplantation

Biomarker	Validation status	Selected references	n	Proposed threshold	Prognostic impact
AFP	Prospectively and externally validated	Vibert et al[63]	153	Δ15 ng/mL/month	Decreased survival
		Lai et al[62]	432		Tumor progression
		Mailey et al[14]	2,253	≥400 ng/mL	Decreased overall survival
		Duvoux et al[24]	972	>1,000 ng/mL	Higher tumor recurrence Microvascular invasion
		Berry and Ioannou[18]	45,267	>65 ng/mL	Higher mortality
DCP	Insufficient external validation	Taketomi et al[43]	90	<300 mAU/mL	Lower recurrence rates
		Takada and Uemoto[44]	136	≤400 mAU/mL	Increased survival
		Kim et al[32]	180	≥200 mAU/mL	Microvascular invasion
		Shindoh et al[39]	124	>450 mAU/mL	
		Ma et al[37]	117	>40 mAU/mL	
AFP-L3	Insufficient external validation	Cheng et al[58]	4,465	No agreement	Decreased survival
OPN	Preclinical stage	Sieghart et al[61]	125	No agreement	Higher recurrence rates
NLR	Insufficient external validation	Halazun et al[45]	150	≥5	Decreased survival
		Limaye et al[46]	160		Higher recurrence rates
		Xiao et al[47]	280	≥4	
CTCs	Insufficient external validation	Xue et al[136]	40	≤5/7.5 mL	Increased recurrence-free survival Lower recurrence rates
		Sun et al[129]	123	≥2/7.5 mL	
cfDNA	Preclinical stage	Ono et al[139]	46	No agreement	Microvascular invasion Decreased recurrence-free survival
		Liao et al[140]	41		

Abbreviations: AFP-L3, *Lens culinaris* agglutinin fraction of alpha-fetoprotein; AFP, alpha-fetoprotein; CTC, circulating tumor cells; DCP, Des-γ-carboxyprothrombin; NLR, neutrophil-to-lymphocyte ratio; OPN, osteopontin; cfDNA, cell-free DNA.

quence, international guidelines have removed serum AFP from the current HCC screening and diagnostic algorithms.[1,12] The actual value of serum AFP relies on its prognostic capacity[10]: increased AFP is associated with histologically poor tumor differentiation, microvascular invasion, increased postoperative tumor recurrence rates and reduced overall survival after LT.[13–17]

A large observational study (n=45,267) showed that patients exceeding Milan criteria had a posttransplant survival comparable to patients who underwent LT for nonmalignant indications if preoperative serum AFP was <15 ng/mL, achieving a 72% 6-year survival rates.[18] Unfortunately, such reduced AFP levels are uncommon among patients outside Milan criteria. A higher threshold of AFP <400 ng/mL may be acceptable to rescue more patients exceeding Milan criteria for LT according to two independent observational studies including 2,419 patients.[14,19] Indeed, pre-LT AFP ≥400 ng/mL is indicative of poor prognosis, doubling posttransplant recurrence rates as well as dropout from the waiting list due to tumor progression or cancer-related symptoms.[14,19–22] Moreover, in clinical practice, patients with AFP levels >1,000 ng/mL should be carefully evaluated and LT may be discouraged because of an unacceptable risk of HCC recurrence, even if they fulfill Milan criteria.[23,24] In some LT institutions, these patients are evaluated as potential candidates only if AFP decreases below 400 ng/mL after local ablation in a stable fashion.[9,25]

Des-y-carboxyprothrombin (DCP)

Also known as prothrombin induced by vitamin K absence II (PIVKA-II), DCP is an abnormal form of prothrombin produced predominantly by HCC cells.[26] According to a meta-analysis of 12 observational studies, DCP has a modest sensitivity (71%) and specificity (84%)[27] for HCC diagnosis,[28] which could be improved in combination with other biomarkers such as AFP.[29–31] Increased DCP also indicates a more aggressive tumor phenotype,[30,32] increased microvascular invasion rates,[17,33–35] extrahepatic metastases[36] and accelerated proliferation.[37]

DCP may be used to predict disease-free survival and overall survival after LT and is widely used in the Eastern world.[38–40] DCP >400 mAu/mL at inclusion on the waiting list was associated with a 5-fold increased risk of posttransplant HCC recurrence.[41] Unfortunately, these results have not been sufficiently validated. In combination with AFP <300 ng/mL, DCP levels <300 mAU/mL result in 5-year recurrence-free survival rates as high as 70%, provided that total tumor diameter was <10 cm, and in the absence of macrovascular invasion or extrahepatic spreading.[42] In Japan, the Kyushu University considered LT in patients with tumors ≤5 cm diameter and serum DCP ≤300 mAU/mL, despite the number of nodules, achieving 5-year overall survival rates of 83%.[43] The Kyoto University proposed to restrict the number of nodules (≤10) while increasing the DCP threshold to ≤400 mAU/mL, again with excellent results: 5-year survival rates of 87% and HCC recurrence rates below 3%.[44]

Inflammatory markers

Neutrophil-to-lymphocyte ratio (NLR),[45–48] platelet-to-lymphocyte ratio (PLR)[49] and, more recently, lymphocyte-to-monocyte ratio (LMR)[50] have been proposed as useful biomarkers to predict posttransplant HCC recurrence, overall survival and/or dropout from the waiting list. Reduced baseline NLR was significantly associated with better overall survival (HR =1.80, $P<0.00001$) and recurrence-free survival (HR =2.23, $P<0.00001$) after LT, according to a meta-analysis of 90 studies including 20,475 patients.[51] Conversely, NLR ≥5 was a significant predictor of poor disease-free survival (HR =19.98, $P=0.005$) in patients within Milan criteria.[45] On the other hand, a high PLR was significantly associated with worse overall survival (HR =1.60, $P=0.0005$) in a meta-analysis of 10 studies involving 2,315 patients.[49] Moreover, a pretransplant PLR ≥125 was associated with significantly more aggressive tumors.[52] A more recent study has addressed LMR in LDLT candidates with HCC, showing that a low LMR may be an independent predictor of worse prognosis, particularly among patients beyond the Milan criteria.[50]

C-reactive protein (CRP) is elevated in many clinical conditions including infections, autoimmune or inflammatory diseases and cancer. In HCC patients, CRP ≥10 mg/dL has been linked with reduced overall survival and recurrence-free survival,[53] and it seems to correlate with vascular invasion rates.[53–56] However, it is unlikely that CRP may become part of therapeutic decisions in HCC patients, either alone or in combination with other biomarkers, given its insufficient specificity.

Other serum markers

The *Lens culinaris* agglutinin fraction of alpha-fetoprotein (AFP-L3) is a variant of AFP based on the sugar chain structure. AFP-L3 is observed mainly in malignant cells[36] and it correlates with tumor size,[57] thus resulting particularly useful in HCC patients who show normal serum AFP. Elevated AFP-L3 was an independent predictor of decreased overall survival and disease-free survival in a meta-analysis of 15 observational studies including 4,465 patients.[58] Unlike other

biomarkers described above, AFP-L3 is not directly associated with vascular invasion, and its potential role to select candidates for LT remains to be explored.

Osteopontin (OPN) is a protein expressed by transformed malignant epithelial cells from several tumors. It has been suggested as an useful independent diagnostic tool in HCC.[59] OPN is over-expressed in advanced tumors, particularly in those with poor histological differentiation,[60] and it has been proposed as an independent predictor of tumor recurrence and survival in patients beyond Milan criteria undergoing LT.[61] Although promising, optimal thresholds remain to be determined and further validated.

Behavior of the HCC on the waiting list

Dynamic changes in AFP

AFP progressive elevation is a surrogate marker of tumor aggressiveness, foreseeing satellite nodules, vascular invasion and extrahepatic spreading even in small uninodular HCC. As a rule of thumb, dynamic changes in AFP are more reliable than a preoperative single value.[18,22] AFP slope >15 ng/mL/month after locoregional ablative therapy (LRT) is an independent predictor of HCC post-LT recurrence,[62] and it is associated with decreased overall survival (77% vs 54% at 5 years; $P<0.0001$)[62,63] and recurrence-free survival (74% vs 47% at 5 years; $P=0.01$).[63] There have been several attempts to include AFP as part of a continuous score for patients with HCC awaiting LT. The *HCC-MELD* score aims to be a continuous score of survival benefit according to liver function and AFP level, calibrated to the survival benefit of non-HCC patients by MELD (for instance, an HCC-MELD score of 20 would calibrate to the survival benefit of a biological MELD score of 20).[64] HCC-MELD was designed as a system to give additional, nonarbitrary points based on transplant survival benefit calculation, and it has been independently validated to predict posttransplantation outcome.[65] Another variation named deMELD score (where "de" stands for "dropout equivalent"), comprising tumor size, number of nodules and AFP value, aims to provide a dynamic and more accurate assessment of dropout on waiting list.[66,67] Further prospective international validation is needed before these scores are implemented in routine clinical practice.

Radiological response to locoregional therapies

The purpose of bridging LRT is to prevent tumor progression and dropout from the waiting list and also to reduce the risk of posttransplant HCC recurrence.[68–70] Radiofrequency ablation (RFA) and trans-arterial chemoembolization (TACE) are the most frequent modalities of LRT, and there is no definitive evidence that one is superior to the other.[71] The use of bridging LRT is highly recommended when the expected length within the waiting list is longer than 6 months,[1] but in clinical practice the vast majority of patients undergo LRT unless technically unfeasible.

In this context, the absence of radiological response to LRT is indicative of increased risk of HCC recurrence after LT.[68,72,73] Successful downstaging has been associated with excellent posttransplantation outcomes, achieving a 4-year intention-to-treat survival over 92%.[74] In a German study assessing TACE for patients awaiting LT (n=50), increased tumor recurrence rates were found in those who showed disease progression despite LRT (64.6% vs 5.5%, $P=0.001$).[75] This is the rationale for the "ablate and wait" strategy, according to which a waiting period of at least 3 months after LRT would be mandatory in order to select those patients with a low risk of recurrence, as a partially treated tumor with favorable biology would not progress in a short observation time.[72,74,76–78] The increasing evidence regarding this issue led to a recent change of the allocation system in the United States, which now incorporates a 6-month delay before granting MELD exception points in order to identify higher-risk HCC patients.[79]

Tissue biomarkers

Liver biopsy is not routinely performed for HCC diagnosis in clinical practice due to the precision of dynamic imaging techniques and to the potential risk of biopsy-related complications including tumor seeding.[80,81] However, the emerging role of tissue biomarkers to assess tumor biological behavior and to predict response to targeted therapies is changing this scenario.

Vascular invasion is a critical hallmark in HCC spreading, both within and outside the liver, and indicates an evolved tumor phenotype. Microvascular invasion is defined as a tumor emboli within a vascular space (either portal or suprahepatic), not detectable by radiological techniques, and forms the most reliable predictor of tumor recurrence, able to double the risk after liver resection and triple the risk after LT.[82] Unfortunately, microvascular invasion may only be evaluated in the whole HCC specimen and cannot be ruled out by conventional needle biopsy. This fact limits its applicability to select candidates for LT. However, patients who undergo liver resection and show microvascular invasion may be considered for LT, given that tumor recurrence would occur in up to 70% of cases otherwise. There are other classical

histological features of HCC related to worse prognosis and able to be detected in a liver biopsy specimen. Moderate–poor tumor differentiation according to the Edmonson scale is associated with more aggressive tumors.[3] The group from the University of Toronto considered patients above Milan criteria for LT without any restriction on number/diameter of nodules if poor differentiation is ruled out in a liver biopsy of the dominant nodule.[83] In addition, certain HCC histological subtypes such as sarcomatoid[84] or macrotrabecular[85] are associated with poorly differentiated grades, higher rates of vascular invasion and reduced overall survival.

Glypican-3 (GPC-3) has been identified as a critical tissue marker of HCC.[86] It is expressed by more than 90% of AFP-negative tumors,[87] while it is not found on cirrhotic and non-cirrhotic adult liver tissues.[88] GPC-3 staining pattern, despite not correlating with tumor differentiation,[88] is an independent prognostic factor for reduced disease-free survival in patients with HCV infection.[89–91] Many other biomarkers have been associated with either poorer survival or aggressive tumor behavior,[92] including lysosomal protein transmembrane 4 beta-35 (LAPTM4B-35),[93] focal adhesion kinase (FAK),[94] fascin (FSCN1),[95] histone deacetylases,[96] CD133,[97] nucleophosmin (NPM),[98] basic leucine zipper transcription factor ATF-like 2 (BATF2),[99] programmed cell death ligands (PDL),[100] E-cadherin,[101] Dickkopf-1 (DKK1)[102,103] and OPN.[104,105] However, the true potential of these biomarkers requires further evaluation.

18FDG-Positron emission tomography (PET)

Liver tissue highlighted by ^{18}FDG displays poor grades of differentiation (RR =9.5) and microvascular invasion (RR =6.4).[106,107] A positive ^{18}FDG-PET has been significantly associated with higher risk of post-LT recurrence (HR =3.95, P=0.024) as well as reduced disease-free survival (87% vs 57% at 3 years, P<0.001).[108–110] In addition, patients outside Milan criteria with a negative PET showed no statistically significant differences in terms of 5-year recurrence-free survival rates when compared to patients within Milan criteria (81% vs 86.2%, P=0.002),[111] while a PET positive became an independent predictor of dropout from the waiting list (HR =5.7, P=0.01), providing additional information to elevated serum AFP.[112]

Liquid biopsy

The detection of tumor byproducts in the bloodstream, also known as liquid biopsy, is a rapidly evolving research field that has engaged the scientific community in recent years. Indeed, identifying circulating-tumor-cells (CTC)[113] or cell-free DNAs (cfDNA)[114] in the peripheral circulation is a noninvasive and reproducible procedure that could potentially provide dynamic information regarding tumor activity.[115–118] The quantitative and qualitative assessment of circulating tumor-derived products is attractive as a research field, not only for screening or diagnosis, but also to select candidates for LT and prioritize them within the waiting list.

Circulating tumor cells

HCC recurrence after LT implies that remnant tumor cells are left behind after surgery and remain in the bloodstream unnoticed to the surveillance of the immune system (which is impaired by the use of immunosuppressive drugs).[119] The assessment of CTCs before LT is an appealing research field with a huge translational potential. Theoretically, patients with persistently increased number of CTCs despite LRT would be removed from the waiting list and offered alternative therapies, and conversely patients with a complete clearance of CTCs after LRT would be perfect candidates to be included within the waiting list and further prioritized irrespective of their tumor burden.

CTCs have been extensively evaluated in different types of cancer. In order to be implemented in HCC clinical practice, detection techniques of CTCs may be sufficiently accurate, reproducible, affordable and without any significant intra- and interobserver variability.[120] In addition, it would be highly desirable that the methodology would allow for an effective recovery of viable CTCs for their ulterior characterization and culture. However, CTCs are scarce in peripheral blood, lost into millions of leukocytes and erythrocytes, thus hampering their identification by using conventional flow cytometry. There is no perfect method for CTCs detection, and usually several technologies are combined to counteract each other disadvantages.[120–122] Two assays have emerged and launched into the market with some supporting clinical evidence for identification, isolation and enumeration of CTCs in HCC, namely, CellSearch® (Veridex Inc.), which is the only one approved by the FDA, and Isoflux (Fluxion Biosciences), which claims for an improved sensitivity[123] and isolation capacity. Both technologies are based on the detection of epithelial cellular adhesion molecule (EpCAM) positive/CD45 negative ($EpCAM^+/CD45^-$) cells. EpCAM is a transmembrane glycoprotein that is present in epithelial cells and in some types of cancer cells, including HCC.[124] In the tumoral tissue, $EpCAM^+$ cells have been correlated with vascular invasion, multinodular tumors and reduced overall survival,[113,125,126] being more frequent in the tumor invasion edge.

In the bloodstream, CTCs are much increased in patients with microvascular invasion, macrovascular invasion and multinodular disease[126] leading to poor overall survival.[127,128] Persistence of CTCs after liver resection has been associated with early HCC recurrence by using the CellSearch System®: a CTCs ≥2 count was identified as an independent prognostic factor in multivariate analysis, even in subgroups with normal AFP and low-risk histological features.[129] Similar findings have been reproduced by other research groups but without an agreement on the optimal threshold of CTCs to predict tumor recurrence.[118,130–132] A CTCs count slope is also associated with disease progression,[133] and the persistence of CTCs after a potentially curative therapy may be an independent prognostic factor.[134,135] In patients undergoing LT, an independent association between recurrence-free survival after LT and preoperative CTCs count was demonstrated.[136] Even in patients with low AFP levels, CTCs count still predicted outstanding recurrence rates.[136] Nevertheless, no significant association was observed between HCC recurrence after LT and total CTCs change or CTCs subtype in another study,[137] probably related to its insufficient statistical power due to the small sample size.

A number of issues remain to be solved before implementing CTCs as a biological marker in HCC patients undergoing LT: 1) different methodologies to detect CTCs may be compared face to face in order to identify the most accurate to become the gold standard; 2) an automated methodology for CTC counting may be implemented to reduce interobserver variability while decreasing enumeration length and 3) new prospective, multicenter and well-designed studies need to be conducted in LT candidates and further independently validated.

Cell-free DNA

Detection of cfDNA is another modality of liquid biopsy that is associated with tumor burden, microvascular invasion and extrahepatic metastasis,[138–140] thus identifying disease progression even in cases of negative AFP. It has been shown to be a highly specific biomarker,[141,142] more frequently detected in patients with larger tumors, poor histological differentiation and associated with reduced overall survival as well as early tumor recurrence after curative resection.[143–145] Moreover, cfDNA allows to analyze HCC genetic and epigenetic profile,[146] as it resembles the matched primary tumor.[140] cfDNA is also easier to obtain after LRT, and its quantification mirrors radiological response as well as disease progression.[139]

However, HCC may suffer heterogenous molecular transformations, even in the presence of similar histopathological findings,[147] which would make it unlikely to identify every relevant mutation in a single blood sample.[148] Targeted deep sequencing may allow to circumvent intratumor heterogeneity,[149] identifying mutations which could act as new therapeutic targets. The expression or absence of cfDNA related to EpCAM and AFP defined up to four HCC subtypes, each one displaying distinct tumor features and reflecting different hepatic lineages, being $EpCAM^{+}/AFP^{-}$ tumors significantly correlated with lower rates of portal vein invasion and improved overall prognosis. Conversely, tumors with cfDNA encoding AFP were linked to worse prognosis.[124] These findings are mainly exploratory but they may impact the decision-making algorithm in the future.

Conclusion

Selected patients outside Milan criteria but showing favorable tumor behavior may be acceptable candidates for LT. Surrogate markers of tumor biology are able to predict outcomes after LT and may be incorporated as part of the decision-making algorithm. Among them, only serum AFP is sufficiently validated to make practical recommendations. Raising AFP over 400 ng/mL despite LRT should motivate a deep evaluation of tumor burden before including a patient in the waiting list. If AFP persists >1,000 ng/mL, LT should be discouraged, even in patients within Milan criteria. There are other serum biomarkers such as DCP, AFP-L3 and NLR, which are already standardized and available in clinical practice, but they deserve further validation to define thresholds and to validate their accuracy before being implemented. Tissue biomarkers are hampered by the need of pre-LT liver biopsy, which is not routinely performed. Liquid biopsy allows detecting and enumerating CTCs, and even to analyze DNA mutations, thus offering direct and dynamic information concerning tumor biological behavior. Although there are still some technical caveats to be solved, liquid biopsy will probably reshape the management of HCC in the upcoming years.

Disclosure

The authors report no conflicts of interest in this work.

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