New Strategies in Hepatocellular Carcinoma: Genomic Prognostic Markers

Augusto Villanueva1,2, Yujin Hoshida3, Sara Toffanin4,5, Anja Lachenmayer4, Clara Alsinet1,2, Radoslav Savic4, Helena Cornella1,2, and Josep M. Llovet1,2,4,6

Abstract

Accurate prognosis prediction in oncology is critical. In patients with hepatocellular carcinoma (HCC), unlike most solid tumors, the coexistence of two life-threatening conditions, cancer and cirrhosis, makes prognostic assessments difficult. Despite the usefulness of clinical staging systems for HCC in routine clinical decision making (e.g., Barcelona-Clinic Liver Cancer algorithm), there is still a need to refine and complement outcome predictions. Recent data suggest the ability of gene signatures from the tumor (e.g., EpCAM signature) and adjacent tissue (e.g., poor-survival signature) to predict outcome in HCC (either recurrence or overall survival), although independent external validation is still required. In addition, novel information is being produced by alternative genomic sources such as microRNA (miRNA; e.g., miR-26a) or epigenomics, areas in which promising preliminary data are thoroughly explored. Prognostic models need to contemplate the impact of liver dysfunction and risk of subsequent de novo tumors in a patient’s life expectancy. The challenge for the future is to precisely depict genomic predictors (e.g., gene signatures, miRNA, or epigenetic biomarkers) at each stage of the disease and their specific influence to determine patient prognosis.

Background

Hepatocellular carcinoma (HCC) accounts for more than 90% of liver cancers, and is a major health problem with 700,000 new cases per year worldwide. Most HCCs arise within a previously damaged liver; chronic hepatitis (B and C) and alcohol abuse being the leading environmental causes for the underlying liver disease (1). Approximately 20 to 30% of the estimated 170 million hepatitis C (HCV)–infected individuals worldwide will develop cirrhosis. The annual incidence of HCC in cirrhotic patients is 3 to 5%, and one third of them will develop a tumor over their lifespan. In addition, because of its high prevalence, hepatitis B virus (HBV) precedes most HCC cases in Africa and Asia, and is the main etiologic factor worldwide. Different strategies aimed at reducing HBV infection, including universal nationwide vaccination programs and antiviral therapies, have shown a significant reduction in the HBV-related HCC burden (2–4).

HCC is the leading cause of death among cirrhotic patients, and the third cause of cancer-related mortality (5). In Western countries, less than 30% of newly diagnosed patients are eligible for curative therapies such as resection, liver transplantation, or local ablation (1). Moreover, 15 to 20% of early stage tumors present with dismal outcome leading to prompt neoplastic dissemination and short-term survival. According to the National Cancer Institute levels of evidence, no level I studies show survival benefits of conventional chemotherapy in HCC patients. However, the unprecedented results of a phase III trial (level IA) showing that sorafenib, a multikinase inhibitor, improves survival in patients with advanced disease, opened a new era in the therapeutic approach to this cancer (6). Results from this trial established sorafenib as the new standard of care, showed the benefits of molecular-targeted therapies, and underscored the importance of oncogene addiction discovery in HCC (7). Unlike other solid tumors (e.g., gastrointestinal stromal tumor), no oncogenic addiction loops were identified in HCC; but, considering the excellent therapeutic results obtained in other addicted tumors, its discovery and selective blockade could significantly impact patient prognosis. This seminal step has changed the landscape of clinical and translational research in the field. A number of molecular compounds are currently moving to late clinical developmental phases, clearly highlighting several unmet needs remaining in the primary and adjuvant settings.
Accurate prediction of patient therapeutic response based on tumor molecular singularities will further improve overall efficacy of molecular therapies in HCC.

Two major advancements could critically improve the outcome of patients with this neoplasm: the first is the identification of critical molecular subclasses with different prognostic implications. Prognosis prediction still relies exclusively on clinical parameters, and molecular data are not guiding therapeutic decision making, which represents a critical bottleneck for improving patient outcome. Identification of biomarkers able to define subgroups of patients with dismal prognosis will translate into better therapeutic strategies and allocation of resources (8). Second, the identification of key genetic or epigenetic drivers of specific subclasses will enable development of more personalized treatment algorithms. Both challenges are hampered by the complexity of the molecular basis of liver cancer. This review briefly analyzes novel advances in genomic-based prognosis assessment in HCC, reviewing miRNA gene signatures reported and summarizing data on microRNA (miRNA) and epigenomic biomarkers. We also present an overview of an integrated model of outcome prediction in HCC, combining clinical and genomic data coded in the tumor- and nontumor-adjacent cirrhotic tissue.

On the Horizon

Modeling prognosis integrating clinical and genomic data

The last decade has witnessed a revolution in the way scientists characterize the human genome. Most of these advances relied on an exponential increase in the throughput capacity of new genomic technologies. Researchers are no more restricted to analyze a limited number of genes, but instead, the whole genome can be thoroughly scrutinized. Moreover, different functional aspects of the genome can be simultaneously evaluated, including DNA structural damage (e.g., point mutations, chromosomal rearrangements), functional genomics (e.g., mRNA and miRNA dysregulation), epigenetics (e.g., aberrant methylation and histone deacetylation), metabolic profiles, etc. The quantity of information generated with these new technologies still surpasses our capacity to assimilate it. This vast amount of molecular data, multidimensional in nature, demands an integrated analytic approach within systems biology frameworks (9). Unlike other malignancies, HCC remains somehow orphan to such innovative and complex investigational initiatives.

Cancer classification aims to establish prognosis, select the adequate treatment for the best candidates, and aid researchers to design clinical trials with comparable criteria. Different staging systems are currently available in HCC, but none has, so far, incorporated molecular data. Cirrhosis, another life-threatening condition, is present in more than 80% of patients with HCC, and renders prognosis prediction a major challenge. Some clinical-based staging systems (e.g., Barcelona-Clinic Liver Cancer (BCLC) algorithm; ref. 7), have addressed both components, establishing a road map for routine clinical decision making. Nevertheless, performance of clinical-based systems requires further refinement to address some daily clinical situations. For example, current systems are unable to accurately detect HCC dropouts from the waiting list for liver transplantation, or to preoperatively identify patients that will develop a tumor recurrence after surgical resection. Inaccurate predictions can cause unnecessary harm to patients, preclude application of curative therapies, and significantly increase healthcare costs.

The unprecedented high-throughput capacity of newly developed array-based genomic platforms favors the assumption that genome-wide approaches could help to improve prognostic estimations achieved by clinical systems. Genomic profiling has already shown its prediction benefits in other malignancies (10, 11). In fact, some signature-based chips are currently under evaluation as predictors of therapeutic response in oncology (e.g., breast). Although initially restricted to fresh-frozen samples, current technologies allow genomic profiling of partially degraded samples, such as formalin-fixed paraffin-embedded tissue (12, 13). In addition, the performance of current array-hybridization–based technologies continues to improve, while their cost decreases (14, 15). These multigene-based assays are now classified according to the U.S. Food and Drug Administration as potential diagnostic devices [in vitro diagnostic multivariate index assays (IVDMIA); ref. 16]. Many studies have proposed molecular classifications of HCC using mRNA-based gene expression profiling, obtained from tumor- or nontumor-adjacent cirrhotic tissue and are reviewed elsewhere (8, 9, 17). Gene signatures from the tumor, capturing biological signals related to proliferation and cell cycling (e.g., "proliferation class," ref. 18; "G3," ref. 19; "class A," ref. 20), seem to identify patients with more aggressive disease. Moreover, patients with tumors supposedly derived from progenitor cells tend to have worse prognosis (e.g., "hepatoblast signature," ref. 21; "EpCAM," ref. 22; "CK19 signature," ref. 23; Table 1; refs. 12, 19–38). In this regard, mRNA profiling seems to indicate tumor cellular lineage. Poor prognostic signatures generated, so far, have not been specifically associated with any risk factor, such as HCV or HBV, or an underlying pre-neoplastic condition. This finding would indicate that genes predicting poor outcome might be common regardless of etiology, an area of research that should be pursued with further studies. Also, genomic profiling of the adjacent nontumoral cirrhotic tissue allowed the development of signatures able to accurately identify patients with poor prognosis (12, 38). This accuracy is probably due to the ability of the signatures to identify the risk of developing de novo tumors, progression of liver dysfunction, and detection of microenvironment-favoring conditions for intrahepatic metastasis. In fact, one of the signatures from adjacent tissue indicating poor prognosis has been recently validated in a different scenario. We tested this signature in a cohort of compensated cirrhotic patients with a median follow-up of 10 years; one...
third of whom died because of liver complications. The poor prognosis gene signature identified 20% of patients at high risk of developing complications (ascites, bleeding, HCC) and poor outcome (39). Thus, this signature identifies the risk of progression of cirrhosis, and might be a relevant tool for trial enrichment in chemopreventive studies. However, all of these signatures were frequently ill defined, and generated in patients at different stages and with distinct etiologies for their underlying liver disease. Hence, they require independent external validation on a patient-by-patient basis. Once validated, the next step will include the design of physical devices (e.g., chips), including the key prognostic genes. These devices will require prospective evaluation in routine clinical conditions prior to their definitive implementation and inclusion in guidelines.

Table 1. Relevant miRNA-based and epigenetic alterations and the prognostic impact in HCC patients to be tested or confirmed

<table>
<thead>
<tr>
<th>Molecular alteration</th>
<th>Clinical significance</th>
<th>REMARK recommendations</th>
<th>Status†</th>
</tr>
</thead>
<tbody>
<tr>
<td>mRNA-based (gene signatures)‡</td>
<td>Poor survival</td>
<td>OK</td>
<td>EV</td>
</tr>
<tr>
<td>Poor-survival signature (186 genes; ref. 12)§</td>
<td>Poor survival</td>
<td>OK</td>
<td>EV</td>
</tr>
<tr>
<td>EpCAM signature (22)</td>
<td>Poor survival</td>
<td>OK</td>
<td>EV</td>
</tr>
<tr>
<td>Venous metastasis signature (38)</td>
<td>Hepatic metastasis</td>
<td>OK</td>
<td>EV</td>
</tr>
<tr>
<td>Class A (20) and/or hepatoblast signature (21)</td>
<td>Poor survival</td>
<td>OK</td>
<td>IV, EV</td>
</tr>
<tr>
<td>G3 subclass (19)</td>
<td>Poor survival</td>
<td>—</td>
<td>IV, EV</td>
</tr>
</tbody>
</table>

miRNA based

<table>
<thead>
<tr>
<th>mRNA-based (gene signatures)‡</th>
<th>Clinical significance</th>
<th>REMARK recommendations</th>
<th>Status†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Down-regulation miR-26a (26)</td>
<td>Poor survival</td>
<td>OK</td>
<td>EV</td>
</tr>
<tr>
<td>20-miRNA signature (31)</td>
<td>Venous metastasis, overall survival</td>
<td>OK</td>
<td>EV</td>
</tr>
<tr>
<td>Down-regulation miR-122 (24)</td>
<td>Poor survival</td>
<td>—</td>
<td>IV, EV</td>
</tr>
<tr>
<td>Down-regulation Let-7 members (25)</td>
<td>Early recurrence</td>
<td>—</td>
<td>IV, EV</td>
</tr>
<tr>
<td>Up-regulation miR-125a (27)</td>
<td>Better survival</td>
<td>—</td>
<td>IV, EV</td>
</tr>
<tr>
<td>19-miRNA signature (30)</td>
<td>Poor survival</td>
<td>—</td>
<td>IV, EV</td>
</tr>
<tr>
<td>Up-regulation miR-221 (28)</td>
<td>Multinodularity; reduced time to recurrence</td>
<td>—</td>
<td>T, IV, EV</td>
</tr>
<tr>
<td>Up-regulation miR-92, miR-20, miR-18 (29)</td>
<td>Poor differentiation</td>
<td>—</td>
<td>T, IV, EV</td>
</tr>
</tbody>
</table>

Epigenetics

<table>
<thead>
<tr>
<th>Molecular alteration</th>
<th>Clinical significance</th>
<th>REMARK recommendations</th>
<th>Status†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genome-wide hypomethylation (32)</td>
<td>Tumor progression, survival</td>
<td>—</td>
<td>IV, EV</td>
</tr>
<tr>
<td>Hypermethylation of E-cadherin or GSTP1 (35)</td>
<td>Poor survival</td>
<td>—</td>
<td>IV, EV</td>
</tr>
<tr>
<td>Degree of hypomethylation (33)</td>
<td>Advanced histologic grade; larger tumor size</td>
<td>—</td>
<td>T, IV, EV</td>
</tr>
<tr>
<td>264-gene hypermethylation profile (34)</td>
<td>Moderately and/or poorly differentiated tumors</td>
<td>—</td>
<td>T, IV, EV</td>
</tr>
<tr>
<td>Hypermethylation of E-cadherin (36)</td>
<td>Microvascular invasion; tumor recurrence</td>
<td>—</td>
<td>T, IV, EV</td>
</tr>
<tr>
<td>Hypermethylation of RIZ1 (37)</td>
<td>Correlation with disease-free survival</td>
<td>—</td>
<td>T, IV, EV</td>
</tr>
</tbody>
</table>

Abbreviations: T, need further preliminary prognostic evaluation; IV, lacks internal validation; EV, lacks external validation.

*Reasonable compliance with REMARK recommendations for tumor marker prognostic studies (44).

†Current status in terms of clinical implementation.

‡Molecular classifications (mRNA-based) with prognostic impact are thoroughly discussed elsewhere (8, 9, 17).

§Genomic signature obtained from early HCC.
concept (12). The scientific challenge is set to identify which is the genomic vector that determines patient prognosis at each stage of the disease. Once discovered, current strategies on treatment allocation, clinical trial design, and chemoprevention will probably require reevaluation. As cancer progresses, genomic data from the tumor will be increasingly informative because, at this point, tumor dissemination will govern patient survival, which has been the case with the majority of gene signatures with prognostic significance reported so far. As depicted in Table 1, most gene signatures have been obtained from tumors resected at intermediate or even advanced tumoral stage, and this explains their prognostic implication.

Lessons learned from genome-based prognostic assessment: Priming study design

Accumulated experiences of translational genomics studies teach us that the reported prognostic signatures are often not reproducible or valid due to problems with study design (e.g., sample size, process of validation, relevance to actual clinical context), or differences in assay platform and/or protocol (40–42). Most of the published microarray studies were conducted on retrospectively collected tissue samples, which were initially nonintentionally obtained for these studies. This collection could result in biased representation of patient population and cause low reproducibility by increasing clinical heterogeneity across studies, which could be further enhanced by small sample size. This factor can be critical in HCC research because considerable diversity exists in demographic and clinical variables, such as etiology, disease stage, or patient ethnicity according to geographic site (43).

Crucial methodologic issues are involved with microarray-based biomarker discovery investigations: (1) estimation of appropriate sample size; (2) adjustment for multiple significance testing to reduce the risk of false discovery; (3) assessment of the statistical confidence of classification or prediction for each patient considering actual clinical application of the biomarker; and (4) validation of the biomarker on a completely independent set of patients, preferably from different institutions. Moreover, calculated sample size may not assure the clinical relevance of the molecular biomarker if the effect size (i.e., extent of differential expression measured as statistic, fold change, etc.) varies across assay platforms. Lastly, all such microarray studies should adhere to the standard requirement for diagnostic

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**Fig. 1.** Model of HCC prognosis combining clinical and genomic data. Stages follow the BCLC algorithm (7). Circles represent an educated guess of the relative influence of each causality component in patient prognosis, at different stages of the disease. In patients with early tumors, survival is mostly determined by genomic data coded in nontumoral cirrhotic tissue ("field effect"), because it determines the risk of liver dysfunction and development of a de novo HCC. As cancer progresses, genomic data from the tumor (in gray) increase its prediction capacity because cancer-related death limits survival in patients with advanced disease.
and/or prognostic biomarker research summarized in the REMARK statement (44). Table 1 summarizes the analysis of gene signatures according to these statements and the type of validation required by them to be incorporated in clinical guidelines. For instance, several biomarker studies are in advanced phases of preclinical development, including mRNA signatures from the tumor (e.g., EpcAM; ref. 22) and the adjacent tissue (e.g., poor survival; ref. 12); although they still require independent external validation for definitive clinical implementation. In terms of miRNA, only one study evaluating miR-26a (26) has reached advanced developmental phases, whereas epigenetic biomarkers are still in exploratory phases.

A key issue when searching for a prognostic biomarker (i.e., “supervised” analysis using outcome information as a guide) is to use a uniform definition for clinical endpoints to enable interstudy comparisons (7). This endpoint is preferably correlated with more homogeneous biological property in order to achieve reproducible results, less affected by variation in clinical practice across institutions. For example, in discovering a tumor recurrence–predictive biomarker, time to recurrence is preferable to disease-free survival, which is the composite of recurrence and death that may not be attributable to a single biological characteristic captured by the biomarker (7). In HCC, it is important to note that two types of recurrence can arise from a distinct biological background: true metastasis as dissemination of primary tumor cells (usually within 2 years after curative treatment) and de novo metachronic tumors occurring in a diseased liver (45). The proportion of each type of recurrence greatly varies across tumor stages (46).

Beyond mRNA-based predictions: Prognostic role of miRNA and epigenetics

Besides mRNA-based gene signatures, recent data suggest the prognostic capacity of genome-wide mRNA and methylation profiling in cancer (47), making it plausible that their predictions will complement those obtained with mRNA arrays. Currently, more than 700 human miRNAs have been discovered and annotated in the miRBase registry (miRBase version 12.0). A growing number of studies indicate that miRNAs are frequently deregulated in cancer. A recent study showed how few miRNAs (approximately 200) accurately reflected the tissue of origin and developmental history of human cancers, and provided a more accurate tumor classification compared with the expression profiling of 16,000 mRNAs (48). Several studies support the ability of miRNA profiling to classify cancer patients according to their clinical outcome [e.g., leukemia ref. 49, colon refs. 50, 51, lung ref. 52, breast refs. 53, 54, and pancreatic ref. 55 cancer]. In HCC, a number of studies have highlighted the prognostic predictive power of miRNA profiling (Table 1). Data coming from these studies, even those at the high-end level, need to be externally validated prior to being incorporated into clinical guidelines. This need is seen in the most relevant study published to date; low expression of miR-26a was reported as an independent predictor of survival in a large cohort of hepatitis B–related Chinese HCC patients (26). Another study showed that the expression of more than 200 precursor and mature miRNAs in HCC and adjacent benign livers provided a 19-miRNA signature significantly associated with disease outcome (30). Similarly, tumors with metastatic HCC had a distinctive 20-miRNA signature compared with nonmetastatic disease after analyzing 241 patients (31). Also, evidence indicates that miR-122 is frequently down-regulated in HCC patients with poor prognosis (24). It seems that the loss of miR-122 could be associated with suppression of the hepatic phenotype and the acquisition of malignant and invasive properties. Down-regulation of members of the Let-7 family, which target important oncogenes such as RAS and MYC, has been found significantly associated with tumor early recurrence (25). Interestingly, these patients were also clustered in a molecular subclass (i.e., “proliferation subclass”), identified upon unsupervised analysis of mRNA-based array data (18). From a therapeutic standpoint, expression restoration of certain miRNA resulted in suppression of cancer cell proliferation, induction of tumor-specific apoptosis, and dramatic protection from disease progression in experimental models (56). This study shows that miRNA modulation is a feasible alternative in HCC therapeutics, desiring initial exploration in the early clinical setting (57). In addition, cholesterol-modified antisense oligonucleotides, termed antagonirs, and locked-nucleic acids have been developed to inhibit the expression of complementary miRNA, and their use in in vitro models is currently under investigation (58).

DNA hypermethylation is a highly prevalent molecular lesion in cancer cells, and it has been long correlated to transcription inactivation. Unlike genetic alterations, certain epigenetic lesions are potentially reversible and lead to heritable states of gene expression that are not caused by changes in the DNA sequence (59). DNA-methylation mapping revealed cancer-specific profiles of hypermethylated CpG islands (hypermethylomes) able to distinguish between different tumor types (60), and to predict patient outcome. Studies reporting epigenetic biomarkers are summarized in Table 1. DNA is methylated by methyltransferases (DNMT), enzymes whose overexpression is associated with poor survival in HCC (61, 62). Furthermore, inactivation of tumor suppressor genes (TSG) involved in cell cycle, apoptosis, and proliferation have been described in HCC. APC, p16, SFRP1, IGFBP3, RASSF1, and SOCS1 show the highest frequency of promoter hypermethylation and consequent gene silencing (32). Additionally, a methylation index of 105 TSGs showed a significant inverse correlation with HCC patient survival (63). A very recent study showed that a methylation profile present in surgical margins of HCC patients treated with resection significantly impacted survival, indicating that epigenetic alterations, similarly to mRNA profiling (12), of nontumor-adjacent tissue is of great potential to predict patient prognosis (37). On the contrary, low methylation levels in certain promoter regions are also linked to advanced histopathologic grade in HCC (33). Pharmacopeigenetics, an emerging
discipline, can eventually aid physicians to anticipate treatment response. For example, hypermethylation of the DNA-repair gene MGMT is the best-known independent predictor of response to chemotherapy in glioblastomas (64). DNA-demethylating agents and histone deacetylase inhibitors (HDACi) are entering clinical practice. Recently, the HDACi suberoyanilide hydroxamic acid has been approved for the treatment of cutaneous T-cell lymphoma (65). However, no single demethylating agent is currently under advanced clinical evaluation in HCC.

In summary, it is difficult to predict which of the above approaches will make the first, significant impact in clinical practice. This difficulty is, in part, due to continued technical improvements of mRNA, miRNA, and epigenetic data collection and analyses. However, it can be expected that integrative analyses of the data obtained with all three approaches will potentially constitute more informative prognostics than either of the approaches alone.

**Future Prospects**

The so-called postgenomics era has highly enriched translational research. Data at the transcriptomic and epigenetic levels are easily obtained from large series of samples, both being great sources for generating hypothesis. More comprehensive studies are now feasible, and tools allow studying biological systems as a whole, far beyond a mere description of its parts. Although the concept is old (i.e., systems biology), the technology necessary to address these studies is gradually becoming accessible. Although now mainly focusing on simple organisms, it is expected that these studies will soon develop a more translational perspective, undertaking clinical practice challenges. In HCC, three areas will substantially benefit from this approach: prognosis assessment, prediction of treatment response, and identification of novel targets for molecular therapies.

In terms of prognosis assessment, recently reported prognostic gene signatures and miRNA can enter and complement clinical variables in staging systems, once they have been externally validated by independent studies. On the other hand, promising data are currently under development with epigenetic biomarkers, which are expected to refine prognostic stratification. Finally, predictors of treatment response will emerge along with novel drugs in the treatment of HCC. Sorafenib’s positive results (6) have opened a new era in HCC research. Future trends in drug development will pivot on accurate assessment of genetic traits in human disease on an individual basis (i.e., personalized medicine). In HCC, the identification of these singularities will allow maximizing therapeutic response by selecting the best drug for the ideal candidate. In this scenario, precise segregation between driver and passenger events is a major challenge. Additionally, personalized approaches may facilitate population enrichment in clinical trials, which could ultimately decrease the futility rate among investigational drugs.

**Disclosure of Potential Conflicts of Interest**

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