New Strategies in Hepatocellular Carcinoma: Genomic Prognostic Markers

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Abstract

Accurate prognosis prediction in oncology is critical. In patients with hepatocellular carcinoma (HCC), unlike most solid tumors, coexistence of two life-threatening conditions such as cancer and cirrhosis difficult prognostic assessments. Despite the usefulness of clinical staging systems for HCC in routine clinical decision-making (e.g., Barcelona-Clinic Liver Cancer algorithm), there is still 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 miRNA (e.g., miR-26a) or epigenomics, areas in which promising preliminary data is thoroughly explored. Prognostic models need to contemplate the impact of liver dysfunction and risk of subsequent de novo tumors in patient’s life expectancy. The challenge for upcoming years will be 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 as the leading environmental causes for the underlying liver disease. Approximately 20-30% of the estimated 170 million hepatitis C (HCV)infected individuals worldwide will develop cirrhosis. The annual incidence of HCC in cirrhotic patients is 3-5%, and one third of them will develop a tumor over their lifespan. In addition, due to its high prevalence, hepatitis B virus (HBV) lies beneath most HCC cases in Africa and Asia, being the main etiological factor worldwide. Different strategies aimed to reduce HBV infection, including universal nationwide vaccination programs and antiviral therapies have demonstrated a significant reduction in the HBV-related HCC burden.

HCC is the leading cause of death among cirrhotic patients, and the third cause of cancer-related mortality. In Western countries, less than 30% of newly diagnosed patients are eligible for curative therapies such as resection, liver transplantation or local ablation. Moreover, 15-20% 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, there are no level I studies demonstrating survival benefits of conventional chemotherapy in HCC patients. However, the unprecedented results of a phase III trial (level IA) showing that sorafenib, a multi-kinase inhibitor, improves survival in patients with advanced disease, opened a new era in the therapeutic approach to this cancer. Results from this trial established sorafenib as the new standard of care, demonstrated the benefits of molecular targeted therapies, and underscored the importance of oncogene addiction discovery in HCC. Unlike other solid tumors (e.g., GIST), there are no oncogenic addiction loops 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: First, the identification of critical molecular subclasses with different prognostic implications. Prognosis prediction still relies exclusively on clinical parameters and molecular data is not guiding therapeutic decision-making, which represents a critical bottleneck for improving patient outcome. Identification
of biomarkers able to define sub-groups of patients with dismal prognosis will translate into better therapeutic strategies and allocation of resources. 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 will briefly analyze novel advances in genomic-based prognosis assessment in HCC, reviewing mRNA gene signatures reported and summarizing data on miRNA and epigenomic biomarkers. We will also present an overview of an integrated model of outcome prediction in HCC combining clinical and genomic data coded in the tumor and non-tumor 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, demand an integrated analytic approach within systems biology frameworks. 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, what renders prognosis prediction a major challenge. Some clinical-based staging systems (e.g., Barcelona-Clinic Liver Cancer (BCLC) algorithm), 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 demonstrated its prediction benefits in other malignancies\textsuperscript{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\textsuperscript{12, 13}. In addition, the performance of current array-hybridization-based technologies continues to improve while their cost decreases\textsuperscript{14, 15}. These multi-gene-based assays are now classified according to the U.S. Food and Drug Administration as potential diagnostic devices (\textit{In vitro} diagnostic multivariate index assays (IVDMIA)\textsuperscript{16}). Many studies have proposed molecular classifications of HCC using mRNA-based gene expression profiling, obtained from tumor or non-tumoral adjacent cirrhotic tissue and are reviewed elsewhere\textsuperscript{8, 9, 17}.

Gene signatures from the tumor capturing biological signals related to proliferation and cell cycling (e.g., “proliferation class”\textsuperscript{18}, “G3”\textsuperscript{19}, “class A”\textsuperscript{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”\textsuperscript{21}, “EpCAM”\textsuperscript{22}, “CK19 signature”\textsuperscript{23}) (Table 1\textsuperscript{12, 19-22, 24-38}). In this regard, mRNA profiling appears 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 underlying pre-neoplastic condition. This 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 non-tumoral cirrhotic tissue allowed the development of signatures able to accurately identify patients with poor prognosis\textsuperscript{12, 38}. This is probably due to their ability to identify risk of developing \textit{de novo} tumors, progression of liver dysfunction and detection of micro-environmental 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 which died due to liver complications. The poor prognosis gene signature identified 20% of patients at high risk of developing complications (ascites, bleeding, HCC) and poor outcome\textsuperscript{39}. Thus, this signature identifies risk of progression of cirrhosis, and might be a relevant tool for trial enrichment in chemopreventive studies. However, all these signatures were frequently ill-defined, 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, next step will comprise 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.

In principle, any genomic-based staging system in HCC should incorporate information related to tumor aggressiveness, hepatic dysfunction, and risk of de novo HCC development. Hence, such prognostic model should consider genomic data coded in the tumor itself and the adjacent non-tumoral cirrhotic tissue. By genomic data we mean an integrative vector obtained upon assimilation of genetic, transcriptomic and epigenetic data. Figure 1 summarizes the core structure of a versatile prognostic model that combines genomic information from each tissue compartment (i.e., tumor and adjacent cirrhosis) at different stages of HCC. In patients with very early HCC (i.e., tumors less than 2 cm without vascular invasion or extrahepatic spread) treated with surgical resection in which tumor is likely removed before dissemination, prognosis will be mostly determined by the risk of developing a de novo primary tumor and the risk of liver dysfunction. Both risks are coded in the surrounding cirrhotic tissue and framed within the “field effect” concept. 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 re-evaluation. As cancer progresses, genomic data from the tumor will be increasingly informative because, at this point, tumor dissemination will govern patient survival. This is the case of the majority of gene signatures with prognostic significance reported so far. As depicted in table 1, most of them 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/protocol. Most of the published microarray studies were conducted on retrospectively collected tissue samples, which were initially non-intentionally obtained for these studies. This 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 can be critical in HCC research since there is considerable diversity in demographic and clinical variables such as etiology, disease stage, or patient ethnicity according to geographic site.
There are crucial methodological issues when dealing 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 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/prognostic biomarker research summarized in the REMARK statement. 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, there are several biomarker studies in advanced phases of preclinical development including mRNA signatures from the tumor (e.g., EpCAM) and the adjacent tissue (e.g., poor survival), although they still require independent external validation for its definitive clinical implementation. Regarding miRNA, only one study that evaluated miR-26a has reached advanced developmental phases whereas epigenetic biomarkers are still on 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 inter-study comparisons. 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 single biological characteristic captured by the biomarker. In HCC, it is important to note that there are two types of recurrence that can arise from 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. The proportion of each type of recurrence greatly varies across tumor stages.

Beyond mRNA-based predictions: prognostic role of miRNA and epigenetics.

Besides mRNA-based gene signatures, recent data suggest the prognostic capacity of genome-wide miRNA and methylation profiling in cancer, being 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. Several studies support the ability of miRNA profiling to classify cancer patients according to their clinical outcome (e.g., leukaemia, colon, lung, breast and pancreatic 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 high-end level, need to be externally validated prior being incorporated into clinical guidelines. This is the case of the most relevant study so far published, low expression of miR-26a was reported as an independent predictor of survival in a large cohort of hepatitis B related Chinese HCC patients. 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. Similarly, tumors with metastatic HCC had a distinctive 20-miRNA signature compared with non-metastatic disease after analyzing 241 patients. Also, there is evidence indicating that miR-122 is frequently down-regulated in HCC patients with poor prognosis. It seems that loss of miR-122 could be associated with suppression of hepatic phenotype and the acquisition of malignant and invasive properties. Down-regulation of members of Let-7 family, that target important oncogenes such as RAS and MYC, has been found significantly associated with tumor early recurrence. Interestingly, these patients were also clustered in a molecular subclass (i.e., “proliferation subclass”) identified upon unsupervised analysis of mRNA-based array data. 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. This study demonstrates that miRNA modulation is a feasible alternative in HCC therapeutics, deserving initial exploration in the early clinical setting. In addition, cholesterol-modified antisense oligonucleotides, termed ‘antagomirs’ and locked-nucleic acids (LNA) have been developed to inhibit the expression of complementary miRNA and their use in vivo models is currently under investigation.

DNA hypermethylation is a highly prevalent molecular lesion in cancer cells and it has been long correlated to transcription inactivation. Unlike genetic alterations, they are potentially reversible and lead to heritable states of gene expression that are not caused by changes in the DNA sequence. DNA-methylation mapping revealed cancer-specific profiles of hypermethylated CpG islands (hypermethylomes) able to distinguish between different tumor types and to predict patient outcome. Studies reporting epigenetic biomarkers are summarized in Table 1. DNA is methylated by methyltransferases (DNMTs), enzymes which over-expression is associated to poor survival in HCC. 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.
Additionally, a methylation index of 105 TSGs showed a significant inverse correlation with HCC patient survival\textsuperscript{64}. A very recent study showed that a methylation profile present in surgically margins of HCC patients treated with resection significantly impacted survival, indicating that epigenetic alterations, similarly to mRNA profiling\textsuperscript{12}, of non-tumor adjacent tissue is of great potential to predict patient prognosis\textsuperscript{37}. On the contrary, low methylation levels in certain promoter regions are also linked to advanced histopathological grade in HCC\textsuperscript{33}. Pharmacoepigeneics, 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\textsuperscript{65}. DNA-demethylating agents and histone deacetylase inhibitors (HDACis) are entering clinical practice. Recently, the HDACi suberoylanilide hydroxamic acid has been approved for the treatment of cutaneous T-cell lymphoma\textsuperscript{66}. However, there is not a single demethylating agent 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 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, being both great sources to generate hypothesis. More comprehensive studies are now feasible, and there are tools that allow studying biological systems as a whole, far beyond a mere description of its parts. Even though this is an old concept (i.e., systems biology), the technology necessary to address these studies is gradually becoming accessible. Despite nowadays it mostly focuses on simple organisms, it is expected that it 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.

Regarding 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 is 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 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, what could ultimately decrease futility rate among investigational drugs.
FIGURE LEGENDS

Figure 1.- Model of HCC prognosis combining clinical and genomic data. Stages follow the BCLC algorithm7. 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 non-tumoral cirrhotic tissue ("field effect") since it determines risk of liver dysfunction and development of a de novo HCC. As cancer progresses, genomic data from the tumor (in grey) increases its prediction capacity since cancer-related death limits survival in patients with advanced disease.
Table 1.- Relevant miRNA-based and epigenetic alterations, which prognostic impact in HCC patients needs 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></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Poor-survival signature (186 genes)†</td>
<td>Poor survival</td>
<td>OK</td>
<td>EV</td>
</tr>
<tr>
<td>EpCAM signature ‡</td>
<td>Poor survival</td>
<td>OK</td>
<td>EV</td>
</tr>
<tr>
<td>Venous metastasis signature §</td>
<td>Hepatic metastasis</td>
<td>OK</td>
<td>EV</td>
</tr>
</tbody>
</table>
| Class A /
Hepatoblast signature ²¹                           | Poor survival                 | OK                      | IV, EV       |
| G3 subclass ²⁹                                            | Poor survival                 | -                       | IV, EV       |
| miRNA-based                                               |                               |                         |               |
| Down-regulation miR-26a ²⁶                               | Poor survival                 | OK                      | EV           |
| 20-miRNA signature ³¹                                     | Venous metastasis, overall survival | OK                    | EV           |
| Down-regulation miR-122 ²⁴                               | Poor survival                 | -                       | IV, EV       |
| Down-regulation Let-7 members ²⁵                         | Early recurrence              | -                       | IV, EV       |
| Up-regulation miR-125a ²⁷                                | Better survival               | -                       | IV, EV       |
| 19-miRNA signature ³⁰                                     | Poor survival                 | -                       | IV, EV       |
| Up-regulation miR-221 ²⁸                                 | Multinodularity; reduced time to recurrence | -                    | T, IV, EV    |
| Up-regulation miR-92, miR-20, miR-18 ²⁹                 | Poor differentiation          | -                       | T, IV, EV    |
| Epigenetics                                               |                               |                         |               |
| Genome wide hypomethylation ³²                          | Tumor Progression, Survival   | -                       | IV, EV       |
| Hypermethilation of E-cadherin or GSTP ³⁵               | Poor survival                 | -                       | IV, EV       |
| Degree of hypomethylation ³³                             | Advanced histological grade; larger tumor size | -                    | T, IV, EV    |
| 264-gene hypermethylation profile ³⁴                    | Moderately/poor differentiated tumors | -                    | T, IV, EV    |
| Hypermethilation of E-cadherin ³⁶                        | Microvascular invasion; tumor recurrence | -                    | T, IV, EV    |
| Hypermethilation of RIZ ³⁷                               | Correlation with disease-free survival | -                    | T, IV, EV    |

* Reasonable compliance with REMARK recommendations for tumor marker prognostic studies ⁴⁴
† Current status in terms of clinical implementation (T: need further preliminary prognostic evaluation, IV: lacks internal validation, EV: lacks external validation)
¶ Molecular classifications (mRNA-based) with prognostic impact are thoroughly discussed elsewhere ⁸, ⁹, ¹⁷
Φ Genomic signature obtained from early HCC
References


Very early
(Stage 0)
Single nodule <2 cm,
no vascular invasion,
PST 0

Early
(Stage A)
Single nodule, 3 nodules <3 cm,
no macrovascular invasion,
PST 0

Intermediate
(Stage B)
Multiple nodules,
no macrovascular invasion,
PST 0

Advanced
(Stage C)
Macrovascular invasion,
extrahepatic spread (N1, M1),
PST 1-2

Clinical stage
(BCLC algorithm)

Relative prognostic impact
of molecular data from tumor
and adjacent tissue

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