

**Molecular Prognosis in Liver Disease**  
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Hepatocellular carcinoma (HCC) is one of the most common malignant tumors worldwide, with over 500,000 cases diagnosed each year[1]. The clinical behavior of HCC is heterogeneous and difficult to predict, depending in part on the severity of the background chronic liver disease in which most of these tumors arise[2, 3]. Most patients present with unresectable disease; for those who undergo resection, recurrence rates can be as high as 50% at two years and life expectancy as short as twelve months[4]. Numerous HCC staging systems have been proposed, but most systems provide only limited prognostic information and fail to reliably stratify patients of all stages and etiologies into meaningful outcome groups[3]. Molecular biomarkers have already entered routine clinical use for tumor prognostication. For example, N-myc gene amplification may be the most important prognostic indicator in neuroblastomas, portending a poor clinical outcome for patients with amplification [5, 6]. Recent explosions in the availability of molecular techniques and insights into the molecular biology of HCC have created an unprecedented opportunity to identify molecular prognostic markers. However, there is currently no molecular prognostication for HCC in use in routine clinical practice.

An ideal prognostic marker provides information about recurrence risk and survival across tumor stages, etiologies, and patient populations. Although potential markers may demonstrate independent prognostic value in multivariate analyses or divide patients with significantly different life expectancies, most fail or are not subjected to rigorous statistical validation to determine whether the proposed marker works for patients other than those from whom the data were derived[7]. The identification of such robust and generalizable prognostic markers has proven difficult in HCC. One major challenge to prognostic marker identification is the complexity of HCC biology, which involves alterations of virtually every major tumorigenic pathway. While some molecular alterations are shared among HCCs, others appear to be related to the specific etiology of the HCC or background liver disease [8-10]. Tumors arising in a background of chronic viral hepatitis differ from those arising in alcoholic liver disease[9], and there are different pathways altered in hepatitis B virus (HBV) versus hepatitis C virus (HCV)-related tumorigenesis[8]. Furthermore, the prognostic utility of a marker may be related to how advanced the tumor is at the time of evaluation. For example, a molecular alteration that occurs early in tumorigenesis may predict early post-resection tumor recurrence but may not provide additional information for patients with unresectable disease. Finally, a clinically useful marker of prognosis should be based on widely available techniques.

**MOLECULAR ALTERATIONS LINKED TO ETIOLOGY: P53**

The tumor suppressor gene p53 is currently the most promising and the most controversial marker of prognosis in HCC. The most frequently altered gene in human

tumors, p53 appears to be closely tied to HCC etiology[11]. A specific p53 gene mutation in codon 249 is found in HCCs associated with exposure to aflatoxin B<sub>1</sub>, a fungal metabolite that contaminates corn, rice, and peanuts in parts of Africa and China[12-14]. The codon 249 mutation is uncommon in areas of low aflatoxin exposure[15, 16]. In patients with chronic HBV infection, the viral-encoded HBx protein binds to and inactivates p53[17]. These two processes may be synergistic in patients with both HBV infection and aflatoxin exposure[12]. The estimated worldwide p53 gene mutation frequency in HCC is around 30%[18], regional mutation frequencies range from 0-75%[19, 20]. Alterations of p53 appear to be a late event in hepatocarcinogenesis associated with tumor progression[21-23].

Since p53 mutation rates vary widely with etiology, population under study, and tumor differentiation, it is not surprising that studies of the prognostic utility of p53 have yielded confusing results. While some authors have found p53 mutation in HCC to be an independent risk factor for recurrence[24-26], shorter overall survival[27], shorter survival after surgical tumour ablation[28], and shorter post-recurrence survival [29], others failed to identify p53 mutation as an independent predictor of outcome[30]. Differing results may be, at least in part, due to differing frequencies of various etiologies in the study populations, as none of these studies were limited to a specific etiology of liver disease nor were the results stratified with respect to etiology. P53 status has also been evaluated using immunohistochemistry, although the biological significance of positive p53 immunostaining is not clear. Correlative studies performed in HCC and other solid tumors indicate that immunohistochemistry (IHC) correlates with but is neither perfectly sensitive nor specific for p53 mutation[31-33]. Positive nuclear staining for p53 protein can reflect mutation-induced lack of degradation but also can be seen in rapidly proliferating cells or neoplastic cells that lack a p53 mutation; p53 mutation, on the other hand, can result in p53 absence[33]. P53 expression by IHC was not predictive of survival in series from China, Spain, Japan and Austria[33-38], but was found to be an independent predictor of survival in a study from Italy[39].

### **MOLECULAR MARKERS OF PROLIFERATION: PCNA and Ki67**

Markers of cellular proliferation have been used for prognostication in several tumors and hold some promise as prognosticators in HCC. A proliferative index can be determined by immunohistochemistry using the monoclonal antibody Ki67 (clone MIB-1), which reacts with the large nuclear Ki67 protein required for cell proliferation[40]. Ki67 is expressed in the G<sub>1</sub>, G<sub>2</sub>, S, and M phases of the cell cycle but is not expressed in resting cells (G<sub>0</sub>). Ki67 expression has been identified as a poor prognostic marker in breast cancer, but studies of Ki67 in other tumors (non-Hodgkin's lymphoma, cervical cancer, and colon cancer) have shown little correlation with outcome[40].

Ki67 IHC has been reported as an independent predictor of rapid tumor recurrence in patients who underwent orthotopic liver transplant [41] and a marker of poor prognosis after early tumor recurrence[38]. However, Ki67 positivity did not correlate with outcome in several series including 81 tumors from Austria[37], 91 tumors in Italian patients[39], or in 20 HCCs from Germany[42]. In benign liver from chronic HBV and chronic HCV patients,

Ki67 positivity correlated independently with transaminase levels (ALT) and etiology of liver disease, suggesting that these factors may be confounding tumor staining patterns[43].

Proliferating cell nuclear antigen (PCNA) is a nuclear protein involved in DNA synthesis and repair. A commonly used marker of cellular proliferation, PCNA identifies cells in the G1/S phase of the cell cycle. PCNA can be also be assayed by immunohistochemistry and is usually scored as a percentage of tumor cells with positive nuclear staining. PCNA is an auxiliary protein for DNA polymerase-delta, and its expression is related to DNA synthesis and cell replication. PCNA expression is reported to predict tumor recurrence, especially for small HCC[44-46], and is associated with venous invasion[45, 46], although others have failed to correlate PCNA expression with outcome[36, 42].

### **ARRAY TECHNOLOGIES: DNA Microarrays**

DNA microarray analyses measure RNA expression levels of thousands of genes simultaneously, generating gene expression *signatures* or *profiles* that can be used to distinguish tumor subtypes with prognostic significance, as has been demonstrated in breast cancer[47]and acute lymphocytic leukemia[48]. DNA fragments mounted onto glass slides are hybridized to tissue sample cDNAs that have been labeled with red or green fluorochromes. Depending on relative amounts of RNA from each sample analyzed, each spot on the slide emits a red or green signal. Genes can then be grouped and compared between samples. Challenges to this approach include analysis of the large body of data and identification of biologically important and predictive genes.

Lee et al. (2004) used gene expression patterns of 91 HCCs to identify subclasses of HCC with different prognoses[49, 50]. Gene expression profiles were identified using DNA microarrays. Two groups of patients differing only in survival were identified. The poorer survival patients (cluster A) survived  $30.3 \pm 8.02$  months, whereas overall survival time for the longer survival patients (cluster B) was  $83.7 \pm 10.3$  months ( $p < 0.0001$ )[49, 50]. Among the 406 “survival genes” identified, the best predictors included genes in the cell proliferation group (including PCNA). Other predictive genes included cell proliferation markers, cell cycle regulators, anti-apoptotic genes, and genes involved in ubiquitination and sumoylation. These findings not only illustrate the predictive power of gene expression profiling but also provide insights into important biologic processes in HCC progression.

### **OTHER POTENTIAL PROGNOSTIC MARKERS**

#### **Telomerase**

Telomerase is a nuclear enzyme that stabilizes and extends telomeres, protecting chromosome ends from degradation. Telomerase is inactive in most normal cells, in which progressive telomere shortening with each cell division leads to replicative senescence. Telomerase activation in stem cells and cancer cells allows for continuous cell proliferation[51]. Telomerase reactivation has also been demonstrated in numerous human cancers including over 80% of liver cancers[52], and telomerase activity in HCC may predict early postoperative recurrence[53-55].

## **Angiogenesis**

Hepatocellular carcinomas are widely regarded as hypervascular tumors that possess a unique vascular architecture to the background liver in which they arise. Angiogenesis is an important and early event in the formation and spread of hepatocellular carcinomas and numerous other tumors. Vascular endothelial growth factor (VEGF) is a potent angiogenic factor that stimulates vessel growth in numerous tumors and is highly expressed in HCC[56, 57]. VEGF expression levels predict poor survival[58], and portal vein invasion and capsular invasion have also been related to angiogenic activity[59, 60]. Vascular invasion is widely regarded as a powerful predictive factor of outcome in HCC.

## **Marker combinations**

Recognition of the heterogeneity of HCC biology has led some researchers to analyze the prognostic power of combinations of molecular markers. Several studies have analyzed markers in combination with p53, including MDM2, zinc-binding protein-89 (ZBP-89), and INK4a-ARF[61-63]. Interestingly, MDM2, a transcriptional target and inactivator of p53, was found to be a better predictor of survival than p53[62]. A more complex predictive system was developed using IHC for 11 different markers (AFP, c-erbB2, c-met, c-myc, HBsAg, HCV, Ki67, MMP-2, nm23-H1, p53, and VEGF)[64]. A regression equation was developed to predict prognosis using weighted values based on the predictive markers, c-myc, VEGF, and Ki67[64]. Unfortunately, the equation was not subjected to validation testing on a new set of patients; it was only tested on the original patients for whom the equation was generated. Furthermore, the use of an image analysis system calls into question the feasibility of the system for widespread clinical use.

## **CONCLUSIONS**

In summary, numerous studies have correlated various molecular markers with recurrence and survival. Unfortunately, conflicting findings, differing methodologies, and inhomogeneous study groups make comparison between studies difficult, and no single marker thus far has been satisfactorily validated in a study group other than for those patients from whom the original data were derived. Due to the heterogeneity of HCC, studies that stratify patients by disease etiology and clinical parameters of liver function are needed to assess how the underlying liver disease affects the markers being tested. In clinical practice, panels of molecular markers may be most informative, and such panels may differ for patients with early, intermediate, and advanced disease.

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