CLINICAL—LIVER

A Hepatocellular Carcinoma 5-Gene Score Associated With Survival of Patients After Liver Resection

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BACKGROUND & AIMS: Due to the phenotypic and molecular diversity of hepatocellular carcinomas (HCC), it is a challenge to determine a patient's prognosis. We aimed to identify new prognostic markers of patients with HCC treated by liver resection. **METHODS:** We collected 314 HCC samples from patients at Bordeaux (1998−2007) and Créteil (2003−2007) hospitals in France. We analyzed the gene expression patterns of the tumors and compared expression patterns with patient survival times. Using the coefficient and regression formula of the multivariate Cox model, we identified a "5-gene score" associated with survival times. This molecular score was then validated in 2 groups of patients from Europe and the United States (n = 213) and China (n = 221). **RESULTS:** The 5-gene score, based on combined expression level of *HN1*, *RAN*, *RAMP3*, *KRT19*, and *TAF9*, was associated with disease-specific survival times of 189 patients with resected HCC in Bordeaux (hazard ratio = 3.5; 95% confidence interval: 1.9−6.6; $P < .0001$). The association between the 5-gene score and disease-specific survival was validated in an independent cohort of 125 patients in Créteil (hazard ratio = 2.3; 95% confidence interval: 1.1−4.9; $P < .0001$). The 5-gene score more accurately predicted patient outcomes than gene expression signatures reported previously. In multivariate analyses, the 5-gene score was associated with disease-specific survival, independent of other clinical and pathology feature of HCC. Disease-specific survival was also predicted by combining data on microvascular invasion, the Barcelona Clinic Liver Cancer classification system, and the 5-gene score in a nomogram. The prognostic accuracy of the 5-gene score was further validated in European and US patients with hepatitis C, cirrhosis, and HCC (overall survival $P = .002$) and in Asian patients with HCC with hepatitis B (overall survival, $P = .02$). Combining the 5-gene score with the expression pattern of 186 genes in corresponding cirrhotic tissues increased its prognostic accuracy. **CONCLUSIONS: The molecular 5-gene score is associated with outcomes of patients with HCC treated by resection in different clinical settings worldwide. This new biomarker should be tested in clinical trials to stratify patients in therapeutic decisions.**

Keywords: Molecular Classification; Liver Cancer; Microarray Analysis; BCLC.

Hepatocellular carcinoma (HCC) represents the third leading cause of cancer-related mortality worldwide.[1] Surgical resection is one of the most used curative treatments for HCC. However, results are impaired by a high recurrence rates (50%−70% at 5 years) and tumor-related death (30%−50% at 5 years).[2,3] Early tumor recurrence within the 2 years after surgery is mainly related to local invasion and intrahepatic metastasis; it correlates with tumor biology.[4] Conversely, late recurrence, occurring beyond 2 years after surgery, is mainly related to de

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Abbreviations used in this paper: BCLC, Barcelona Clinic liver cancer; CTNNB1, catenin (cadherin-associated protein) β 1; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; RT-PCR, reverse transcriptase polymerase chain reaction.

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novo tumor formation, coded in the surrounding cirrhotic nontumor tissue due to the "carcinogenic field effect."[4,5] In clinical settings, prognosis assessment and decision of surgical treatment are based on one of the tumor staging systems (ie, Barcelona Clinic liver cancer [BCLC], cancer of the liver Italian program, Japan Integrated Staging, and TNM).[2,6] These different staging systems are based mainly on the tumor size, number of nodules, and severity of the liver disease.[6] Some authors have proposed to improve the staging system by introducing tumor biomarkers, such as the level of α-fetoprotein in serum and pathological features, like microvascular invasion and tumor differentiation.[2,7]

To refine prognosis scoring, the search of molecular biomarkers is an expanding field.[5,8–11] However, the heterogeneity of the underlying liver diseases and tumor stage in the area of HCC has challenged the use of molecular classification in different clinical settings worldwide. More than 18 different molecular signatures have been published, but few have been externally validated.[5,10–13] One of these validated molecular prognostic classifications was the G3 signature, which has been shown to be associated with tumor recurrence in both fresh-frozen and paraffin-fixed HCC.[9,14] Interestingly, the G3 subgroup of HCC also showed the strongest association with tumor recurrence among 18 different molecular signatures.[14] Concerning the cancer field effect in cirrhosis, a 186-gene signature derived from nontumor liver sample was also able to predict late recurrence and survival by capturing biological signals of aggressive phenotype from the underlying cirrhosis.[5,15]

However, a simple, easy to use test, validated in different patients populations, and using various techniques remains to be identified and endorsed in HCC clinical guidelines. We aimed to identify a molecular signature able to accurately predict clinical outcomes of patients with curative surgical resection of HCC. We addressed several key points to reinforce the robustness of our molecular score: (1) identification in a training set of patients, validation in an independent cohort; (2) assessment of the added value of molecular score compared with classical clinical, pathological, immunohistochemical, and molecular markers; and (3) external validation by another group. Finally, we constructed a composite nomogram to combine clinical and pathological with molecular scoring to refine prognosis assessment and overpass the dichotomy between molecular and clinical/pathological prognostic markers.

Materials and Methods

Patients and Tissue Samples

Patient and tumor features are detailed in Table 1 and Supplementary Table 1. HCC samples were systematically frozen after liver resection for tumor in 2 French University hospitals in Bordeaux (from 1998 to 2007) and Créteil (From 2003 to 2007). This study was approved by our local Institutional Review Board committee (CCPRB Paris Saint Louis, 1997 and 2004). All patients gave their informed consent according to French law. We excluded tumors with necrosis >80%, tumors with RNA of poor quality or of insufficient amount, HCC with noncurative resection (R1 or R2 resection or extrahepatic metastasis at the time of the surgery), HCC treated by liver transplantation, and HCC patients dying within the first month after surgery because of surgical complications and/or decompensated cirrhosis (n = 10). Three hundred and fourteen HCCs were qualified for the prognosis analysis (see flowchart in Figure 1 and Supplementary Figure 1). Tumor and nontumor liver samples were frozen immediately after surgery and conserved at −80°C. Tissue samples from the frozen counterpart were also fixed in 10% formaldehyde, paraffin-embedded, and stained with H&E and Masson's trichrome. The diagnosis of HCC was based on established histological criteria.[16] All tumors were reviewed independently by 2 expert pathologists (JC and PBS) without knowledge of the patient's outcomes and initial diagnosis. In case of disagreement regarding the diagnosis or pathological features of HCC, sections were re-examined and a consensus was reached and used for the study. In case of multiple tumors, we have taken into account the diameter of the largest nodule in patient annotation and we have also analyzed the largest nodule at the molecular level.

External Cohorts of Validation

We used 2 independent cohorts of HCC for external validations in the prognostic study (Figure 1 and Supplementary Table 2): HCC genomic consortium[14] (n = 213, GSE20238-GPL570, GSE20238-GPL8432, GSE20140, GPL5474) and the National Cancer Institute cohort (n = 221, GSE14520).[8,12]

Selection of Genes

We selected 103 genes for the quantitative reverse transcriptase polymerase chain reaction (RT-PCR) analysis in the whole study. Using the Affymetrix microarray E-TABM-36, we analyzed the pattern of expression of the 44 HCCs treated by curative resection and public annotations were updated for prognostic data. We performed Cox univariate analysis according to disease-specific survival (ie, death related to the tumor, see end point for prognosis) and tumor recurrence to select the genes significantly associated with death and tumor relapse. After these analyses, we assembled a panel of 41 genes, the most differentially expressed (significance and fold change) between patients characterized by radically different prognosis. In addition, we included 2 genes from, the literature (KRT19 and EPCAM), 2 typical markers related to prognosis and stem cells features in HCC.[13,17] A total of 43 genes were selected for their association with HCC prognosis (Supplementary Table 3). We also selected 60 genes for molecular diagnosis of benign and malignant liver tumors using Affymetrix HG133A gene chip TM microarray hybridizations performed on the same platform (E-TABM-36, GSE7473, GSE11819, and GSE9536; Supplementary Table 4). Identification of molecular diagnostic predictors will be detailed in another study (manuscript in preparation).

Quantitative RT-PCR

RNA extraction and quantitative RT-PCR were performed as described previously.[9] Expression of the 103 selected genes was analyzed in duplicate in all the 314 HCC samples using TaqMan Microfluidic card TLDA (Applied Biosystems, Carlsbad, CA) gene expression assays as described in Supplementary Tables 3 and 4. Gene expression was normalized with the RNA ribosomal 18S, and the level of expression of the tumor sample was compared with the mean level of the corresponding gene expression in normal liver tissues, expressed as an n-fold