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Prognostic Gene-Expression Signature for Patients with Hepatitis C-Related Early-Stage Cirrhosis

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Abstract

Background & Aims—Liver cirrhosis affects 1%–2% of population and is the major risk factor of hepatocellular carcinoma (HCC). Hepatitis C cirrhosis-related HCC is the most rapidly increasing cause of cancer death in the US. Non-invasive methods have been developed to identify patients with asymptomatic, early-stage cirrhosis, increasing the burden of HCC surveillance, but biomarkers are needed to identify patients with cirrhosis who are most in need of surveillance. We investigated whether a liver-derived 186-gene signature previously associated with outcomes of patients with HCC is prognostic for patients newly diagnosed with cirrhosis but without HCC.

Methods—We performed gene expression profile analysis of formalin-fixed needle biopsies from the livers of 216 patients with hepatitis C-related early-stage (Child-Pugh class A) cirrhosis who were prospectively followed for a median of 10 years at an Italian center. We evaluated whether the 186-gene signature was associated with death, progression of cirrhosis, and development of HCC.

Results—Fifty-five (25%), 101 (47%), and 60 (28%) patients were classified as having poor-, intermediate-, and good-prognosis signatures, respectively. In multivariable Cox regression modeling, the poor-prognosis signature was significantly associated with death ($P=.004$), progression to advanced cirrhosis ($P<.001$), and development of HCC ($P=.009$). The 10-year rates of survival were 63%, 74%, and 85% and the annual incidences of HCC were 5.8%, 2.2%, and 1.5% for patients with poor-, intermediate-, and good-prognosis signatures, respectively.

Conclusions—A 186-gene signature used to predict outcomes of patients with HCC is also associated with outcomes of patients with hepatitis C-related early-stage cirrhosis. This signature might be used to identify patients with cirrhosis in most need of surveillance and strategies to prevent their development of HCC.

Keywords

liver cancer prevention; early detection; screening; whole genome gene expression profiling

Liver cirrhosis represents the terminal stage of many chronic fibrotic liver diseases, and is estimated to affect 1–2% of the world's population.^{1, 2} Chronic infection with hepatitis C, afflicting 170 million individuals, is increasingly the cause of cirrhosis together with alcohol abuse in developed countries, and had superseded HIV as a cause of death in the U.S. by 2007.³ Cirrhosis-related mortality is high, with deaths attributable to either cirrhosis-associated complications such as gastrointestinal bleeding or to hepatocellular carcinoma (HCC) that occurs in one third of cirrhotic patients.⁴ Even after complete surgical resection or local ablation of early HCC tumors, most patients develop subsequent *de novo* tumors due to a cancer-prone microenvironment in the cirrhotic liver referred to as the “field effect”.⁵

With the development of non-invasive imaging and laboratory tests such as ultrasound-based liver stiffness measurement, cirrhotic patients have been increasingly diagnosed at early stage and subjected to regular surveillance for HCC.⁶ Clinical management of this growing patient population poses a challenge for cost-effective allocation of medical resources.⁷ In addition, a number of chemopreventive strategies are being explored to abrogate the lethal complications of cirrhosis which include hepatic decompensation and HCC.^{1, 2, 8, 9} Such interventions are often accompanied by significant toxicity, and are

expensive.^{10, 11} Hence, biomarkers identifying patients at highest risk among those with early-stage cirrhosis would be extremely useful not only to enable cost-effective tumor surveillance, but also to prioritize patients for chemopreventive intervention.⁹

Clinical staging systems such as the Child-Pugh classification¹² and Model for End-stage Liver Disease (MELD) score¹³ are only able to distinguish advanced-stage from early-stage cirrhosis. In early-stage cirrhosis, only a handful of laboratory variables (e.g. serum albumin, bilirubin, and platelet count) may carry prognostic information. However, their prognostic capability is limited because the measurement is generally within normal range and its smaller dynamic range is more affected by non-specific fluctuation.¹ Similarly, newer imaging- and laboratory test-based approaches have limited sensitivity for the detection of fibrotic progression and/or fibrogenic/carcinogenic activity in the cirrhotic liver.^{2, 14} Thus, there is a pressing need for robust and sensitive prognostic biomarkers for patients with early-stage cirrhosis.

We recently reported a 186-gene-expression signature in liver tissue that was predictive of overall death in mostly hepatitis C-infected cirrhotic patients who had surgically treated primary HCC.¹⁵ Molecular pathway analysis suggested that the signature reflects the “field effect” in cirrhotic liver. Therefore, in the present study, we hypothesized that the 186-gene signature might be a sensitive predictor of future risk of poor outcome in newly diagnosed patients with hepatitis C-related, early-stage (Child-Pugh class A) cirrhosis who never experienced HCC development nor any of the complications of cirrhosis at the time of diagnosis. Importantly, patients with such early-stage liver cirrhosis, which is far more prevalent than hepatocellular carcinoma, lack effective predictors of clinical outcome. To test our hypothesis, we evaluated the ability of the signature to predict clinical outcome from needle liver biopsies obtained from an independent cohort of 216 patients with hepatitis C-related, Child-Pugh class A cirrhosis who were prospectively followed for a median of 10 years in the context of a HCC surveillance program.^{16–18} (prospective-retrospective design proposed to facilitate biomarker development¹⁹) We also evaluated the signature in multiple assay platforms to assess its clinical applicability.

METHODS

Patients

Patients diagnosed with histologically confirmed liver cirrhosis lacking evidence and history of hepatic decompensation or HCC were enrolled between 1985 and 1998, and prospectively followed for the development of hepatic decompensation, hepatocellular carcinoma, or death as previously described (“**Italian cirrhosis cohort for HCC surveillance**”).^{16–18}

Abdominal ultrasound and esophago-gastroduodenoscopy were performed before the enrollment. Needle biopsy specimens of the liver were obtained within 2 years prior to enrollment, and archived as formalin-fixed, paraffin-embedded tissue blocks. The patients received prospective follow-up every 6 months (see Supplementary Information for details of patient enrollment and follow-up). Among the 360 enrolled cohort patients with various etiologies including viral hepatitis and alcohol abuse, 216 patients with hepatitis C-related, Child-Pugh class A cirrhosis were analyzed in this study (Figure 1). The study was approved by the Review Board of each participating institution on the condition that all samples were anonymized.

Gene expression profiling

Whole-genome gene-expression profiling was performed using the cDNA-mediated Annealing, Selection, extension, and Ligation (DASL) DNA microarray assay (Illumina) as

previously described²⁰ (Supplementary Information). Microarray data are available at NCBI Gene Expression Omnibus (GSE15654).

Statistical analysis

Outcome prediction was performed using the 186-gene signature as previously described (see Supplementary Information for details).^{15, 21} The log-rank test and Cox regression modeling were used to evaluate association of the signature and clinical variables with time from the enrollment to overall death, liver-related death, occurrence of hepatic decompensation (gastrointestinal bleeding, ascites, or hepatic encephalopathy), progression of Child-Pugh class, and development of hepatocellular carcinoma. Liver-related death was defined as death due to liver failure and/or HCC progression. Analyses were performed using either the GenePattern analysis toolkit²² (www.broadinstitute.org/genepattern/) or the R statistical package (www.r-project.org).

RESULTS

Needle biopsy expression profiling

Because the standard approach to assessing cirrhosis in the clinical setting involves needle biopsies followed by formalin fixation, we first sought to assess the feasibility of performing genome-wide expression profiling on such small samples (typically 10 mm × 1 mm pieces of tissue). We previously showed that it is feasible to profile the expression of ~ 6,000 transcripts in large formalin-fixed, paraffin-embedded specimens obtained from surgical resection. Here, we tested ability of the assay to profile expression of all ~ 24,000 protein-coding genes in the human genome in fixed, needle biopsy specimens. Of 280 patients with hepatitis C-related, Child-Pugh class A cirrhosis, 236 patients with sufficient amount of formalin-fixed, paraffin-embedded tissue blocks were subjected to the gene-expression profiling. Among them, 216 (92%) yielded high quality genome-wide expression profiles (Supplementary Figure 1). Clinical demographics of the patients were not changed by the exclusion of poor quality profiles (Supplementary Table 1). While not perfect, this result was remarkable because of (1) the tiny size of the specimens, (2) the age of the archived specimens (up to 23 years old), and (3) the fact that the samples were not collected with gene-expression profiling as a primary goal.

Patient characteristics

Table 1 summarizes clinical characteristics and events of the 216 patients analyzed. After a median follow-up of 10 years (IQR: 7–11 years), 66 patients (31%) died: 28 patients died of hepatocellular carcinoma, 20 patients died of liver failure, 5 patients died of cardiovascular causes, 10 patients died of other non-liver-related causes, and the remaining 3 patients who were lost from follow-up and died of unknown causes. Seventy-one patients (34%) developed hepatic decompensation that required hospitalization during follow-up including gastrointestinal bleeding (n=22), ascites (n=62) and/or hepatic encephalopathy (n=10). Child-Pugh class progressed to B or C in 66 patients (31%). HCC developed in 65 patients (30%) with an annual incidence of 2.9%, which is consistent with prior studies⁴. Twelve patients received liver transplantation (see Supplementary Information for details of outcome assessment).

There was no obvious difference in clinical characteristics or outcomes of the patients in this cohort compared to other published cohorts of hepatitis C-related, Child-Pugh class A or compensated cirrhosis (Supplementary Table 2). The prevalence of gastro-esophageal varices, annual incidence rates of death, hepatic decompensation, and HCC development, proportion of the patients who had the clinical events (i.e., death, hepatic decompensation, and HCC development), and proportion of liver-related deaths were indistinguishable from

prior reports. Specifically, well-established clinical prognostic variables (including age, presence of varices, platelet count, and serum albumin and bilirubin) were similarly prognostic in our dataset (Supplementary Table 3), further indicating that our study cohort is representative of the clinical course of patients with hepatitis C-related, early-stage cirrhosis.

Evaluation of 186-gene signature

We next sought to determine whether the clinical outcome among the cirrhotic patients could be predicted based on the expression pattern of the 186-gene signature (Supplementary Table 4). Importantly, we applied the survival signature to the cirrhosis cohort without modification, thereby precluding any over-optimization of the signature to the present dataset. Using this signature, 55 (25%), 101 (47%), and 60 (28%) patients were classified as having the poor, intermediate, or good prognosis, respectively. Presence of the poor-prognosis signature was associated with poor overall death ($P=.005$), liver-related death ($P=.005$), progression of Child-Pugh class ($P<.001$), and HCC development ($P=.01$) in univariate analysis (Supplementary Table 3). The signature was marginally associated with development of hepatic decompensation ($p=0.06$). Annual death rates were 4.6%, 1.5%, and 1.1% for patients with poor-, intermediate-, and good-prognosis signatures, respectively. Ten-year survival rates were 63%, 74%, and 85% for patients with poor-, intermediate-, and good-prognosis signatures, respectively (Figure 2, A). Ten-year HCC development rates were 42%, 28%, and 18%, and annual rates of HCC development were 5.8%, 2.2%, and 1.5% for patients with poor-, intermediate-, and good-prognosis signatures, respectively (Figure 2, B). These results indicate that the 186-gene signature is capable of stratifying hepatitis C-related, Child-Pugh class A cirrhosis into prognostic subgroups. We note that the signature appears more sensitive than other clinical measures of liver damage such as liver transaminase and alcohol consumption (Supplementary Table 3), and there was no statistically significant association between the prediction and liver transaminase, alcohol intake, and history of interferon-based therapy (Supplementary Information). While this is prognostic stratification within the earliest-stage cirrhosis (Child-Pugh class A) in which clinical prognostic factor is lacking, the magnitude of prognostic separation is well comparable to that in comparison with higher Child-Pugh classes, i.e., B (moderate cirrhosis) and C (severe/end-stage cirrhosis). Furthermore, we could not identify any prognostic gene-expression signature in the cirrhosis data set that outperformed the 186-gene signature (Supplementary information).

To evaluate potential clinical applicability of the signature as a prognostic test, we implemented the 186-gene signature on an independent assay platform that is particularly well-suited to simple, clinical implementation (nCounter assay, NanoString, Inc.). We re-analyzed a subset of samples using the nCounter assay and found that the original result was perfectly recapitulated by the new method (Supplementary Figure 2). The ability of the signature to remain predictive when implemented on independent assay platforms is a strong sign of the signature's robustness (Supplementary Information).

Multivariable analysis

We next examined the value of the signature in the context of clinical variables associated with outcome. Multivariable analyses showed that the poor-prognosis signature remained significant for the association with overall death ($P=.004$), liver-related death ($P=.003$), progression of Child-Pugh class ($P<.001$), and HCC ($P=.009$) (Table 2). The signature was also significantly associated with composite endpoint of death and liver transplantation ($P=.007$) (Supplementary Table 5, 6) and showed trend of association with the development of hepatic decompensation (Supplementary Table 3, 7). The prognostic association was independent from hepatitis C virus genotype 1b, a well-known determinant of anti-viral effect of interferon, and history of interferon treatment (Supplementary Table 3, 8). Presence

of both poor-prognosis signature and high bilirubin level increased the hazard ratio for overall death to 5.78, suggesting that these variables carry complementary prognostic information (Supplementary Table 9), although the prognostic association of bilirubin at the cut-off value needs to be validated in future studies. These strong associations with outcome indicate that the signature retains value as an independent prognostic indicator within Child-Pugh class A cirrhosis, in which clinical predictors of outcome are limited.

DISCUSSION

We have demonstrated that a 186-gene signature is associated with long-term outcomes of patients with hepatitis C-related, early-stage (Child-Pugh class A) cirrhosis for whom effective predictors of outcome are lacking. In contrast to other experimental biomarkers that have been evaluated only for association with short-term outcome measures such as fibrosis progression,^{9, 14, 23} our results are notable for the long follow-up period of the cohort (a median of 10 years) that enabled the assessment of long-term outcomes including patient death.

We observed that our previously defined 186-gene signature was correlated with poor prognosis in 25% of the patients with cirrhosis across the analyzed endpoints including overall death, liver-related death, progression of Child-Pugh class, and HCC development. Importantly, our results indicate that the 186-gene signature is a sensitive measure of cirrhosis severity and lethality even within Child-Pugh class A patients. These observations suggest that the signature predicts the propensity of a patient's cirrhosis to worsen. With such worsening come the well-recognized complications of cirrhosis including the development of hepatocellular carcinoma. This model contrasts with one in which the signature reflects a pre-malignant state *per se*. The close relationship between HCC and cirrhosis-related liver failure is similarly reflected by the historic Child-Pugh classification, which was originally developed as a predictor of survival after transection of bleeding esophageal varices, but which turned out to also predict poor outcome of patients with cirrhosis and/or HCC.²⁴

Of particular clinical relevance is our demonstration that genome-wide expression profiling can be performed on needle liver biopsies obtained during routine clinical care. We had previously shown that such profiling was possible from large, surgical resection specimens, but the feasibility of needle biopsy profiling suggests that the measurement of the survival signature and other such signatures could be implemented in a routine clinical setting. We note that while our predictive signature comprises only 186 genes, it may still be useful to continue to perform genome-wide profiling in the clinical setting because (1) microarray-based genome-wide profiling assays are already commercially available and inexpensive (see also Supplementary Information), (2) sequencing-based transcriptome profiling is likely to be a practical option of clinical test in near future, and (3) such genome-wide data can facilitate the validation of future biomarkers as they are discovered by others.

The mechanism by which cirrhotic progression increases risk of cancer remains to be fully elucidated, but there is some evidence that certain pathways such as the nuclear factor kappaB pathway are involved in the response to liver injury and fibrogenesis, as well as carcinogenesis, and our signature indeed reflects activation of such pathways (Supplementary Tables 10, 11, Supplementary Figures 3, 4). In addition, a component of the signature reflects activation of hepatic stellate cells, known to be a major driver of liver fibrogenesis and supposedly carcinogenesis.^{15, 25, 26} Furthermore, it has been previously reported that genetic polymorphisms in the epidermal growth factor (EGF) gene predispose to hepatocellular carcinoma development.^{27, 28} Indeed, EGF is a component of our signature, and animal models suggest that both the signature and the cirrhotic phenotype are reversible

with EGF receptor inhibitor treatment²⁹ (see accompanying in-submission article by **Fuchs, et al.**). Taken together, these results suggest that the 186-gene signature may provide not only a prognostic biomarker for the identification of high-risk cirrhosis patients, but it may also serve as a pharmacodynamic biomarker of therapeutic response.

It seems likely that cirrhosis patients who harbor the poor-prognosis signature would be most likely to benefit from therapeutic intervention (e.g. anti-cirrhotic agents or chemopreventive strategies).⁹ The potential public health impact of a test that identifies high-risk patients with a disease as prevalent as cirrhosis cannot be overemphasized. Interventions could be focused on those most likely to benefit, and toxicity could be spared for those patients with a low probability of cirrhosis-related morbidity or mortality. For example, a simple Markov model based on using the 186-gene signature to prioritize patients for intervention suggests that the size of a clinical trial of such therapy (and hence its cost and potential for toxicity) could be substantially reduced by enrolling only the high-risk patients to assign either treatment or control arm (Supplementary Table 12, Supplementary Figure 5).^{30, 31} This is of particular importance in an era where numerous agents are being developed with a goal of preventing cirrhosis progression and HCC development.^{2, 8, 9}

An additional obstacle to the clinical development of new anti-cirrhosis treatments is the lack of an effective method to monitor the drugs' effects in patients.^{2, 8} While non-invasive fibrosis-monitoring approaches have the advantage of high rates of compliance,^{2, 8} such features are not at present sensitive enough to detect subtle, early changes in liver fibrosis, or to detect molecular fibrogenic/carcinogenic activity within the liver.¹⁴ As such, they may have limited utility, especially in patients with early-stage cirrhosis. And again, we note that none of these methods have been correlated with long-term clinical outcome as we have demonstrated for the 186-gene signature in the present study.

The incidence of HCC has continued to increase in developed countries. For example, the incidence tripled in the US between 1975 and 2005, and assumed to increase in the next few years and remain high for the next two decades, resulting in an estimated \$1 billion in health care cost.^{32–35} However, implementation of tumor surveillance program, which enables efficient allocation of limited medical resources, is still suboptimal; only 17% of HCC patients are diagnosed at early stage through regular surveillance.³⁶ Therefore, there would be enormous benefit from the establishment of risk-adjusted surveillance approaches.³⁷ Current clinical guidelines recommend tumor surveillance for patient populations with annual incidence exceeding 1.5%.³⁸ It is noteworthy that patients in our study with the good-prognosis signature developed HCC at a rate of 1.5% per year, which is at the threshold triggering the surveillance, whereas patients with the poor-prognosis signature had a nearly four-fold higher rate of tumor development. A cost-effectiveness analysis indeed suggested that use of a signature-adjusted surveillance program may be expected to increase life expectancy with minimal impact on the healthcare cost (Supplementary Table 13). At a minimum, the presence of the poor-prognosis signature would support stricter adherence to the standard surveillance schedules.

Our study suggests that the 186-gene signature is a promising tool for the prediction of long-term clinical outcome in patients with hepatitis C-related, early-stage liver cirrhosis. Although the findings and endpoints should be further validated in independent studies, the introduction of such a test in the clinical setting could have a major impact on approaches to tumor surveillance, patient-enrichment strategies for chemoprevention studies, and on the measurement of the therapeutic effect of emerging anti-cirrhosis therapies. We therefore believe that the signature should be incorporated into future clinical studies of cirrhosis

natural history and therapeutic intervention. Whether the test will have similar utility in non-hepatitis C-related cirrhosis (e.g. hepatitis B or alcohol) remains to be established.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

EGF	epidermal growth factor
DASL	cDNA-mediated Annealing, Selection, extension, and Ligation
HCC	hepatocellular carcinoma
MELD	Model for End-stage Liver Disease

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Figure 1. Study design

Needle liver biopsy specimens collected from the prospectively followed patient cohort were subjected to whole-genome gene-expression profiling.

A

No. at risk

Good prognosis	60	51	30	4
Intermediate prognosis	101	87	49	10
Poor prognosis	55	42	23	3

B

No. at risk

Poor prognosis	55	37	18	3
Intermediate prognosis	101	82	45	8
Good prognosis	60	49	28	3

Figure 2. Probabilities of survival and HCC development

Curves are shown for (A) overall death and (B) HCC development according to the level of expression of the 186-gene signature among all 216 patients. Time 0 indicates the time of enrollment.

Table 1

Characteristics of patients at the time of enrollment and clinical outcomes

Characteristic at enrollment	n=216
Age, median (IQR), y	59 (54–64)
Men, No. (%)	116 (54%)
Esophageal/gastric varices, No. (%)	52 (25%)
Stage F1/F2/F3, No.	42/9/1
Albumin, median (IQR), g/dL	4.1 (3.9–4.4)
Bilirubin, median (IQR), mg/dL	1.0 (0.7–1.2)
Prothrombin time - international normalized ratio, median (IQR)	1.0 (0.98–1.10)
Platelet count, median (IQR), /mm ³	105,000 (79,000–142,000)
Alanine amino transferase, median (IQR), IU	108 (63–167)
Alpha-fetoprotein, median (IQR), ng/mL	6 (3–11)
Hepatitis C virus genotype 1b, No. (%)	122 (58%)
Clinical outcome	
Deaths, No. (%)	66 (31%)
Hepatic decompensation *, No. (%)	71 (34%)
Gastrointestinal bleeding, No.	22
Ascites, No.	62
Hepatic encephalopathy, No.	10
Progression of Child-Pugh class, No. (%)	66 (31%)
HCC, No. (%)	65 (30%)

* Gastrointestinal bleeding, ascites, and/or hepatic encephalopathy.

HCC, hepatocellular carcinoma

Table 2

Association of 186-gene signature and clinical variables with clinical outcome (multivariable analysis)

Variable	Hazard ratio (95% CI)	p value
Overall death		
186-gene signature		
- Poor-prognosis signature *	2.77 (1.38 – 5.57)	.004
- Intermediate-prognosis signature *	1.28 (0.66 – 2.51)	.47
Bilirubin 1.0 mg/dL	2.69 (1.55 – 4.68)	<.001
Platelet count < 100,000 /mm ³	2.47 (1.40 – 4.35)	.002
Liver-related death		
186-gene signature		
- Poor-prognosis signature *	3.76 (1.56 – 9.09)	.003
- Intermediate-prognosis signature *	1.72 (0.73 – 4.00)	.21
Bilirubin 1.0 mg/dL	3.17 (1.62 – 6.21)	<.001
Platelet count < 100,000 /mm ³	2.60 (1.35 – 5.02)	.004
Progression of Child-Pugh class		
186-gene signature		
- Poor-prognosis signature *	3.79 (1.82 – 7.91)	<.001
- Intermediate-prognosis signature *	1.55 (0.75 – 3.19)	.22
Bilirubin 1.0 mg/dL	3.09 (1.76 – 5.44)	<.001
Platelet count < 100,000 /mm ³	2.12 (1.24 – 3.63)	.006
Development of HCC		
186-gene signature		
- Poor-prognosis signature *	2.65 (1.28 – 5.51)	.009
- Intermediate-prognosis signature *	1.60 (0.81 – 3.18)	.18
Bilirubin 1.0 mg/dL	2.33 (1.38 – 3.92)	.001

* Compared to good-prognosis signature.

HCC, hepatocellular carcinoma