Molecular signature predictive of long-term liver fibrosis progression to inform anti-fibrotic drug development

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Abstract

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Data accession number in public database

NCBI Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/): GSE85550.

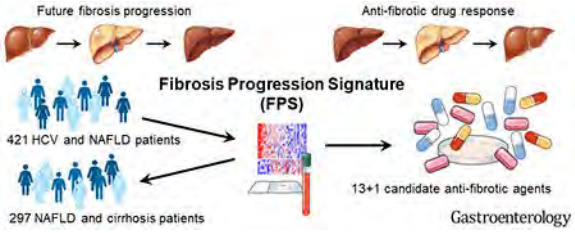
Background/Aims: There is a major unmet need to assess the prognostic impact of anti-fibrotics in clinical trials due to the slow rate of liver fibrosis progression. We aimed to develop a surrogate biomarker to predict future fibrosis progression.

Methods: A Fibrosis Progression Signature (FPS) was defined to predict fibrosis progression within 5 years in HCV and NAFLD patients with no to minimal fibrosis at baseline (n=421), and validated in an independent NAFLD cohort (n=78). The FPS was used to assess response to 13 candidate anti-fibrotics in organotypic ex vivo cultures of clinical fibrotic liver tissues (n=78), and cenicriviroc in NASH patients enrolled in a clinical trial (n=19, NCT02217475). A serum-protein-based surrogate FPS (FPSec) was developed and tested in a cohort of compensated cirrhosis patients (n=122).

Results: A 20-gene FPS was defined and validated in an independent NAFLD cohort (aOR=10.93, AUROC=0.86). Among computationally inferred fibrosis-driving FPS genes, *BCL2* was confirmed as a potential pharmacological target using clinical liver tissues. Systematic ex vivo evaluation of 13 candidate anti-fibrotics identified rational combination therapies based on epigallocatechin gallate, which were validated for enhanced anti-fibrotic effect in ex vivo culture of clinical liver tissues. In NASH patients treated with cenicriviroc, FPS modulation was associated with 1-year fibrosis improvement accompanied by suppression of the E2F pathway. Induction of PPARα pathway was absent in patients without fibrosis improvement, suggesting benefit of combining PPARα agonism to improve anti-fibrotic efficacy of cenicriviroc. A 7-protein FPSec was associated with development of decompensation in cirrhosis patients.

Conclusion: FPS predicts long-term fibrosis progression in an etiology-agnostic manner, which can inform anti-fibrotic drug development.

Graphical Abstract



Keywords

Prognostic prediction; liver fibrosis; drug development; companion biomarker

INTRODUCTION

The liver is one of the major organs affected by fibrosis due to chronic infection of hepatotropic viruses, e.g., hepatitis B virus (HBV) and hepatitis C virus (HCV), and metabolic disorders, e.g., alcohol-associated liver disease (ALD) and non-alcoholic fatty liver disease/non-alcoholic steatohepatitis (NAFLD/NASH). Cirrhosis is the terminal stage of progressive liver fibrosis, affecting 1% to 2% of the global population and causing 1 million deaths annually worldwide, with >50% increase over the past three decades.[1] Cirrhosis is the major predisposing factor for liver cancer, the fourth leading cause of cancer

death worldwide[2, 3] Given the limited survival benefit and high costs of currently available treatment options at advanced stages of the disease, prevention of fibrosis progression at earlier stages is an urgent unmet need to effectively improve the poor prognosis.[3] Fibrosis progression toward cirrhosis typically takes two to three decades.[4] Therefore, it is practically difficult or infeasible to clinically confirm prognostic benefit of experimental anti-fibrotic therapies. Thus, reliable surrogate biomarkers predictive of long-term fibrosis progression will allow estimating clinically meaningful prognostic benefit of anti-fibrotic therapies within the timeframe of typical therapeutic clinical trials. In addition, such prognostic biomarkers will enable enrichment of clinical trial population to improve statistical power to detect therapeutic efficacy.

In patients with established cirrhosis, we previously identified a transcriptomic Prognostic Liver Signature (PLS), which predicts future development of hepatic decompensation, hepatocellular carcinoma (HCC), and death over up to 23 years of clinical observation.[5–7] PLS captures dysregulation of fibrogenic molecular pathways such as transforming growth factor β (TGF-β), epidermal growth factor (EGF), nuclear factor κB (NF-κB), and tumor necrosis factor (TNF) pathways, linked with activation of hepatic stellate cells into myofibroblasts, the major driver of hepatic fibrogenesis.[6, 8] The PLS-based prognostic predictions were consistent across multiple sites in the liver, unlike histological fibrosis staging in liver biopsy tissue.[5] More recently, we observed that PLS predicts future disease progression even after fibrosis was reduced by anti-viral therapies.[9–11] These observations led us to hypothesize that hepatic transcriptome signatures such as PLS may predict fibrotic disease progression at early disease stages before developing substantial fibrosis. Such molecular signatures may serve as surrogate endpoints to gauge prognostic impact of experimental anti-fibrotic therapies. To test the hypothesis and determine utility of such biomarker for anti-fibrotic drug development, we first evaluated the prognostic capability of PLS in liver disease patients with no to minimal fibrosis, and defined the Fibrosis Progression Signature (FPS) as a potential companion biomarker for anti-fibrotic therapies.

MATERIALS AND METHODS

Patients and specimens

Archived formalin-fixed liver tissues from index biopsy were used for histological assessment in all patients (Figure 1 and Supplementary table 1) to confirm no to minimal fibrosis (METAVIR fibrosis stage F0 or F1). The PLS validation set 1 (and FPS derivation set 1) is a case-control series of 43 chronic hepatitis C patients from a prior cohort study[12] consecutively diagnosed and followed at Johns Hopkins and Massachusetts General Hospital between 1998 and 2010. Twenty-five patients were co-infected with HIV and on anti-retroviral therapies.[13] The patients were regularly followed up with ultrasound elastography at median interval of 1.1 (IQR: 0.6-2.0) years. Liver stiffness measurement > 7.0 and > 9.5 kPa were regarded as indication of F2 and F3 fibrosis, respectively.[14, 15] The PLS validation set 2 (and FPS derivation set 2) is a case-control series of 38 patients who consecutively underwent liver transplantation for HCV-related cirrhosis and protocol liver biopsies at year 1, 2, and 5 after transplantation (and additional biopsies as needed to evaluate graft rejection, which were excluded) at Baylor University between 2002 and 2007.

Median number of biopsies was 8 (IQR: 6-9) per patient with median interval between serial biopsies of 8.6 (IQR: 0.3-12.6) months. The cases showed F-stage increase of two stages or more within 5 years of follow-up, and the controls were defined as patients who were free from the fibrosis progression for 5 years or more and matched for sex, age (at 5-year interval), and F-stage at baseline. The FPS derivation set 3 is a cross-sectional series of 31 NAFLD patients who underwent diagnostic liver biopsy at Hiroshima University between 2003 and 2015. The FPS derivation set 4 is a cross-sectional series of 309 NAFLD patients who underwent diagnostic liver biopsy (F0 or F1 fibrosis) at Massachusetts General Hospital between 2009 and 2016. The FPS validation set 1 for this study's primary endpoint, fibrosis progression of one stage or more, is comprised on a case-control series of 78 NASH patients with F1 to F3 fibrosis in index liver biopsy who had a follow-up biopsy to investigate histological disease progression at median interval of 2.4 (IRQ: 2.2-3.0) years at Hiroshima University between 2004 and 2018.[16] The cases were defined as patients who had F-stage increase of one stage or more in the follow-up biopsy. The FPS validation set 2 includes 78 patients with fibrotic liver diseases from various etiologies, for which de-identified fresh liver tissues were available from standard-care hepatic resection for organotypic ex vivo tissue culture at University of Texas Southwestern and Mount Sinai. The FPS validation set 3 consists of NASH patients with F1-F3 fibrosis who underwent liver biopsy before and after 1-year treatment with cenicriviroc (n=9) or placebo (n=10) in the phase IIb CENTAUR trial (NCT02217475).[17] Decrease of one or more fibrosis stage was regarded as anti-fibrotic response. The serum surrogate FPS was assessed in archived de-identified serum samples from 79 patients with chronic liver diseases.[11] The FPSec validation set is a cohort of 122 patients with compensated (Child-Pugh class A) cirrhosis patients with mixed etiologies enrolled at University of Michigan between 2004 and 2006 as reported in our previous study.[7] Hepatic decompensation was defined as newly developed massive ascites, hepatic encephalopathy, bleeding from gastroesophageal varices, or liver transplantation. The study was approved by institutional review board at respective institutions with written informed consent or exemption for use of archived de-identified samples (protocol numbers: STU062018-058, STU072018-071, 2010P000220/PHS, HS13-00159).

Ex vivo and in vitro assessment of pharmacological effects of candidate anti-fibrotic agents

We evaluated galunisertib, erlotinib, AM095, bortezomib, pioglitazone, metformin, epigallocatechin gallate (EGCG), I-BET 151, JQ1, captopril, nizatidine (Selleck Chemicals); MG-132 (Sigma-Aldrich); cenicriviroc (AbbVie) (Supplementary table 2) in organotypic ex vivo culture of precision-cut liver slice (PCLS) tissues in the FPS validation set 2 as previously described.[9] Patient-derived liver cell spheroids were generated from a cirrhosis patient and high-risk FPS was induced by free fatty acids,[18] and treated with EGCG, bortezomib, cenicriviroc, and/or bezafibrate for 48 h. Mycoplasma-free human hepatic myofibroblast cell lines, LX-2 and TWNT-4, were cultured with MG-132 (20 μM) or DMSO control for 12 and 24 h in triplicates.

Immunostaining

Immunostaining was performed[19] for caspase-3 (Asp175) (5A1E, Cell Signaling), alpha-SMA (Abcam), Desmin (DAKO), GFAP (abcam), and Ki-67 (Abcam). TUNEL staining was performed using ApopTag Peroxidase In Situ Apoptosis Detection Kit (EMD Millipore).

Gene and protein expression profiling

Total RNA was isolated from fixed tissue sections by using High Pure RNA Paraffin kit (Roche), and assessed for quality by qRT-PCR of *RPL13A* (Supplementary methods).20 Total RNA from the PCLS tissues was isolated using RNeasy kit (Qiagen). RNA samples (100-200 ng) were subjected to the PLS/FPS assay implemented in the nCounter platform (NanoString), and transcriptome profiling of the CENTAUR trial samples was performed by RNA-Seq (TrueSeq RNA Access, Illumina).[6, 9] Expression of *BCL2*, *COL1A1*, and *ACTA2* genes was measured by qRT-PCR (Supplementary table 3).[21] Serum protein profiling was performed by using xMAP assay (Luminex).[11]

Bioinformatic and statistical analyses

FPS was defined as a subset of PLS specifically associated with time to fibrosis progression and with shared transcriptional regulation between viral (HCV, n=81) and metabolic (NAFLD, n=340) etiologies using Fisher's inverse chi-square statistic (Supplementary methods, Supplementary figure 1). Prognostic prediction was performed by using Nearest Template Prediction algorithm,[6] and association with the clinical outcome was evaluated with uni/multivariable logistic regression. Modulation of gene signatures and molecular pathways was assessed by gene set enrichment index (GSEI), and co-expression gene networks were defined by MEGENA.[9] Rational combination anti-fibrotic therapies were computationally explored based on combinatorial enhanced modulation of the FPS from high- to low-risk pattern in the transcriptome data of the clinical PCLS tissues cultured with the candidate anti-fibrotic agents. Datasets are available at NCBI Gene Expression Omnibus (GSE85550).

RESULTS

PLS is associated with 5-year fibrosis progression in chronic hepatitis C with no or minimal fibrosis

To clarify whether a hepatic transcriptome signature can predict long-term fibrosis progression in early-stage fibrotic liver disease, we analyzed the PLS in 43 patients with F0 or F1 fibrosis in the index liver biopsy (PLS validation set 1), among which 12 patients showed F-stage increase of two stages or more within 5 years (Supplementary table 1). The PLS profiles classified the patients into high- (n=14, 33%), intermediate- (n=12, 28%), or low- (n=17, 40%) risk group for fibrosis progression (Figure 2A). The PLS prediction was significantly and independently associated with histological fibrosis progression in multivariable logistic regression adjusted for clinical confounding variables: ALT and platelet count (adjusted odds ratio [aOR], 10.86; 95% confidence interval [CI], 1.13 - 104.83), and with an area under the receiver operating characteristic curve (AUROC) of 0.81 (Figure 2B and C, Supplementary tables 4 and 5). In this nested case-control

series, 24 patients had HIV co-infection, a known fibrosis accelerator.[22, 23] HIV co-infection showed a trend of association with fibrosis progression, although insignificant likely due to the use of anti-retroviral therapies and limited sample size (univariable OR = 3.20; 95% CI 0.73 - 14.12). The number of patients with active HCV infection has been declining with the widespread use of direct-acting antivirals (DAA), but this result demonstrates a proof of concept that hepatic transcriptome can predict long-term fibrosis progression in a major chronic liver disease etiology, sharing multiple molecular mechanisms of fibrogenesis with other etiologies.[8]

PLS is associated with 5-year fibrosis progression after liver transplantation

The association of PLS with fibrosis progression was further validated in another clinical scenario, liver transplantation. Fibrosis progression after transplantation due to recurrent HCV infection has been the major problem that limits patient survival.[24] Sustained virologic response (SVR) to anti-HCV therapies improves surrogate indicators of fibrosis such as liver stiffness in short-term, which is followed by gradual regression of histological fibrosis.[23, 25] However, SVR rate with DAA post transplantation can be as low as 50% and adverse event rates can be as high as 75% when progressed to decompensated liver disease.[26] Therefore, prediction of fibrosis progression will remain relevant in a subset of post-transplant patients with HCV infection. To evaluate PLS for its capability to estimate risk of future fibrosis progression, we analyzed liver biopsy tissues obtained one year after receiving liver transplantation in 38 HCV cirrhosis patients with F0 or F1 fibrosis, including 21 fibrosis progressors and 17 non-progressors (PLS validation set 2). The PLS profiles classified the patients into high- (n=13, 34%), intermediate- (n=9, 24%), or low- (n=16, 42%) risk groups (Figure 2A). The presence of a high-risk PLS was significantly associated with histological fibrosis progression in multivariable logistic regression adjusted for clinical confounding variables (aOR, 26.50; 95% CI, 1.97 - 355.61) and with an AUROC of 0.87 (Figure 2B and C, Supplementary tables 4 and 5). Thus, the association of PLS with histological fibrosis progression was successfully validated in two clinical scenarios, i.e., chronic hepatitis and post transplantation, in patients with no to minimal fibrosis.

FPS shared between viral and metabolic liver disease etiologies was defined

With the encouraging validation of the PLS in patients with early-stage liver disease, indicating that the hepatic transcriptome informs future progression of fibrotic liver disease, we next sought to define a molecular signature more specifically associated with fibrosis progression. Progressive fibrosis is a common feature shared among viral and metabolic liver disease etiologies.[8] Consistent with the notion, our PLS predicts adverse outcomes in patients with advanced liver diseases caused by viral and metabolic etiologies.[5–7, 9–11, 20, 27] To define a transcriptomic signature associated with long-term fibrosis progression in an etiology-agnostic manner, we integrated the FPS derivation sets 1 to 4, representing major viral (HCV) and metabolic (NAFLD) etiologies (421 patients in total) (Figure 1, Supplementary table 1), for association with time to fibrosis progression and transcriptomic co-expression shared between HCV and NAFLD (Supplementary figure 1A, B, Supplementary methods). We identified a 20-gene FPS, consisting of 14 high- and 6 low-risk genes (Figure 3A, Supplementary table 6). Some of the FPS member genes, e.g., *CCL21* and *LOXL2*, were individually implicated in liver fibrogenesis in chemical

and physiological liver fibrosis models as well as fibrotic liver disease patients,[8] supporting the validity of our approach to identify molecular drivers of liver fibrosis relevant to broad biological and clinical contexts.

FPS-based prognostic prediction is correlated with PLS-based prediction, while it is not completely overlapping, particularly in patients with a metabolic etiology (concordance rates are 72%, 82%, 74%, and 62% in the FPS derivation sets 1, 2, 3, and 4, respectively) (Supplementary figure 1C). The proportion of high-risk prediction is smaller for FPS (14%) compared to PLS (24%) among the patients in the four FPS derivation sets, suggesting that FPS identifies a subset of high-risk PLS patients with elevated risk of fibrosis progression. In the FPS derivation set 1, 13 patients were predicted as having a high-risk of disease progression by the PLS, among which 5 patients (38%) showed 5-year fibrosis progression. Among 11 patients predicted as having a high-risk of disease progression by the FPS, 5 patients (45%) showed 5-year fibrosis progression (Supplementary figure 1D). In the FPS derivation set 2, 13 patients were predicted as having a high-risk of disease progression by the PLS, among which 12 patients (92%) showed 5-year fibrosis progression. Among 11 patients predicted as having a high-risk of disease progression by the FPS, 10 patients (91%) showed 5-year fibrosis progression (Supplementary figure 1E). Furthermore, in multiple independent patient cohorts representing diverse liver disease etiologies, namely HBV, HCV, ALD, and NAFLD (Supplementary table 7), FPS genes were associated with fibrotic liver disease severity and adverse outcomes (Figure 3B). These results collectively warranted further independent validation of FPS for fibrosis progression.

FPS predicts fibrosis progression in NAFLD

To validate FPS for its association with fibrosis progression, we profiled liver biopsy tissues from an independent cohort of 78 NAFLD patients (FPS validation set 1). FPS classified the patients into high- (n=15; 19%), intermediate- (n=44; 56%), and low- (n=19; 24%) risk groups (Figure 3C). Changes in histological fibrosis were assessed in follow-up biopsy performed with a median interval of 2.4 years (IQR: 2.2 - 3.0 years). A high-risk FPS at baseline was significantly associated with the primary endpoint of this study, i.e., progression of fibrosis stage by one or more (aOR, 10.93; 95% CI, 1.11 - 107.78; P = .04), as well as no fibrosis regression (aOR, 13.66; 95% CI, 1.28 – 145.29) (Figure 3D, Supplementary table 4). A high-risk PLS showed association with fibrosis progression (OR, 3.67; 95% CI: 0.57 - 23.47) and no fibrosis regression (adjusted OR, 6.83; 95% CI, 1.04 - 44.87) to lesser extent compared to FPS, suggesting superiority of FPS in estimating risk of fibrosis progression. AUROCs of high-risk FPS are > 0.86 for fibrosis progression and no fibrosis regression, supporting its predictive performance (Figure 3E). The FPS risk predictions changed in the follow-up biopsy along with the F-stage from the baseline, while the predictions were generally correlated (Supplementary figure 1F).

We next assessed whether change in FPS status over the course of clinical follow-up is associated with changes of histological and/or clinical features. We observed that the FPS change was closely correlated with time-adjusted change in histological fibrosis stage along with Mallory body (Figure 3F). The second closest features include hepatic steatosis, hepatocyte ballooning, and histological/biochemical inflammation as well as BMI We also

observed weak correlations with glucose-metabolism-related features (HbA1c, fasting blood glucose, and glycogenated nuclei) and LDL cholesterol. This result suggests that FPS reflects dynamic change in fibrotic, steatotic, and inflammatory histological features in NAFLD liver.

There is a clinical need for biomarkers to detect presence of substantial fibrosis for indication of possible medical intervention. In the FPS validation set 1, there was a trend of association for high-risk FPS with ≥ F2 fibrosis, but not statistically significant (Supplementary figure 1G).

BCL2 is a clinically-relevant pharmacological anti-fibrosis target encoded in the FPS

With the validated association of FPS with fibrosis progression, we next sought to determine whether FPS member genes/proteins represent clues to anti-fibrotic targets, for which FPS serves as a companion biomarker. We first developed co-expression gene networks by integrating the FPS derivation sets 1 to 4 using the MEGENA algorithm as we previously described,[9] and inferred which FPS member genes likely have regulatory role (as fibrosis risk driver genes) to shape the fibrogenesis-promoting hepatic transcriptome (Figure 4A, see Supplementary methods). One of the driver genes, B-cell lymphoma 2 (BCL2), was reported to be over-expressed in human cirrhotic livers and its genetic knockdown with siRNA sensitized myofibroblasts to apoptosis in cell culture experiment.[28] We observed induction of co-regulated genes with BCL2 and suppression of apoptosis pathway in myofibroblasts in single-cell transcriptome profiles of human cirrhotic livers[29] (Figure 4B). We observed the same trends in mouse liver cell transcriptome profiles (Supplementary figure 2).

To test whether BCL2 activation in myofibroblasts can be pharmacologically inhibited as a clinically available anti-fibrotic strategy, we first computationally screened a collection of transcriptomic perturbations by 19,811 bioactive agents (CMap database).[30] We identified several compounds that mimic global transcriptomic modulation by *BCL2* gene knockdown, including MG-132 and bortezomib, which are known as proteasome inhibitors (Supplementary table 8). These compounds indeed reduced myofibroblast activation and inhibit biliary fibrosis induced by bile duct ligation in mice.[31] We validated the effect of MG-132 in human myofibroblast cell lines, LX2 and TWNT4, for suppression of type I collagen (*COL1A1*) and α-smooth muscle actin. (*ACTA2*), hallmarks of hepatic fibrogenesis along with *BCL2* (Figure 4C). Furthermore, we confirmed that MG-132 reduced expression of the genes in organotypic ex vivo culture of human precision-cut liver slice (PCLS) from two patients with fibrosis caused by HCV (F1) and NAFLD (F2) (Figure 4C), accompanied with apoptosis induction shown by increased cleaved-caspase-3-positive cells (Figure 4D) along sinusoidal area where α-smooth muscle actin (α-SMA) is present (Figure 4E). Cleaved caspase-3 was co-localized with a stellate cell marker, glial fibrillary acidic protein (GFAP) in the MG-132-treated PCLS tissue compared to DMSO-treated tissue (Figure 4F). Furthermore, in the clinical fibrotic tissues, ex vivo treatment with MG-132 significantly suppressed the high-risk FPS genes (false discovery rate [*FDR*] ≤ .008), supporting the role of BCL2 in regulating high-risk FPS genes in human fibrotic liver (Figure 4G). These data collectively suggest that FPS provides clues to clinically relevant

anti-fibrotic targets, and serves as a readout to monitor effect of candidate anti-fibrotic agents in patient-derived fibrotic liver tissues.

FPS-based systematic ex vivo assessment of clinical liver tissues identifies combination anti-fibrotic therapies

Multiple candidate anti-fibrotic targets/agents have been proposed in experimental studies,[8] but their clinical relevance is unclear without evaluation in liver disease patients. The FPS modulation by BCL2 inhibition in clinical PCLS suggests that FPS can serve as a readout to evaluate clinically relevant anti-fibrotic effect in pre-clinical models. To systematically explore the idea, we assessed a set of experimental anti-fibrotic agents in ex vivo culture of PCLS tissues (with preserved multi-cell-type tissue microenvironment) from 78 chronic liver disease patients. The tested agents include various classes of compounds: inhibitors of fibrogenic cellular signaling, i.e., TGF-β pathway (galunisertib), epidermal growth factor (EGF) pathway (erlotinib), and lysophosphatidic acid (LPA) pathway (AM095); CMap-derived BCL2 antagonists (MG-132, bortezomib); a dual C-C chemokine receptor type 2/5 (CCR2/CCR5) inhibitor evaluated for treatment of NASH fibrosis (cenicriviroc); anti-diabetics that suppress fibrogenesis as one of their pleiotropic effects (pioglitazone, metformin); a green tea catechin shown to inhibit liver fibrosis in our recent pre-clinical study (epigallocatechin gallate [EGCG]); epigenetic modulators of PLS (I-BET 151, JQ1)[32]; CMap-derived PLS-modulating generic drugs (captopril, nizatidine) (Supplementary table 2).[3, 9, 32–35] After 24h of culture with the agents or vehicle controls, we examined reduction of FPS-based prognostic risk level, i.e., suppression of the high-risk genes and/or induction of low-risk genes jointly quantified as Combined Enrichment Score (CES). The CES-based FPS response was observed in 31% to 88% of the patients for the agents treated in more than five patients (Figure 5A, Supplementary table 9), suggesting that clinical response is heterogeneous across patients and the ex vivo assessment may inform clinical response to the agents. At the drug level, modulation of each individual FPS gene (target FPS gene) varies across the agents, while the targeted genes are similar among subsets of the agents, suggesting that the agents elicit anti-fibrotic effect via shared or unique targets in the FPS (Figure 5B).

The target FPS genes are shared among agents in the same class of compounds such as MG132 and bortezomib, eliciting similar suppression of high-risk FPS genes, *CCL21, BCL2*, and *IGFBP6*. In contrast, agents with distinct mechanism of action such as galunisertib, AM095, and metformin showed similarly striking suppression of *SLC7A1* (also known as *CAT1*), a recently identified anti-fibrotic target.[36] These results demonstrate that the molecular-signature-based systematic evaluation enables unbiased identification of anti-fibrotic agents and their specific targets. In addition, the diverse target FPS genes across the agents suggest opportunities of combining multiple agents that complementarily target FPS member genes for synergistic and enhanced anti-fibrotic effect. To test this idea, we computationally inferred synergistic effect of the agents to shift the FPS from high- to low-risk pattern, i.e., suppression of the high-risk genes and/or induction of the low-risk genes (see Supplementary methods). We identified four candidate combinations based on EGCG as a backbone (Figure 5C, D). The predicted combinatorial anti-fibrotic effect was validated in ex vivo culture of a PCLS tissue from a chronic hepatitis C

patient with F2 fibrosis. We observed that addition of bortezomib and MG132, but not metformin, to EGCG resulted in substantially reduced expression of genes encoding extracellular matrix proteins (e.g., *COL1A1, HAS2*) and fibrogenic drivers (e.g., *PDGFRB, TGFB1, NOTCH1, LPAR1*), confirming their synergistic anti-fibrotic effect in patient-derived fibrotic liver tissue (Figure 5E). The high-risk FPS genes were more broadly suppressed with the combinations compared to mono-therapies (Supplementary figure 3). These results collectively demonstrate that FPS-based analysis of clinical PCLS tissues enables ex vivo testing of candidate anti-fibrotic agents in clinical liver tissues and identifies rational, molecular-targeted combinatorial anti-fibrotic therapies. We further confirmed that combination of EGCG and bortezomib resulted in suppression of broader fibrosis-related genes compared to mono-therapies in a patient-derived liver spheroid (Figure 5F). We recently developed a PLS-inducible cell culture model (cPLS system) for high-throughput screening of HCC chemopreventive agents.[37] We confirmed that pharmacological FPS modulation similar to that in the PCLS culture was observed in the simple and robust cell culture system (Figure 5G), indicating that FPS-based high-throughput compound screening is feasible to efficiently identify new anti-fibrotics with prognostic impact quantitatively measured by FPS modulation.

FPS and global transcriptome profiles to monitor anti-fibrogenic activity of cenicriviroc in NASH patients

In the recent phase IIb CENTAUR trial, 1-year treatment with a dual CCR2/CCR5 inhibitor, cenicriviroc, resulted in improved histological fibrosis that persisted another year in NASH patients with F1 to F3 fibrosis.[17] We profiled the hepatic transcriptomes from these patients to analyze the therapeutic modulation of FPS and global molecular pathways using paired pre- and post-treatment biopsy tissues from 9 cenicriviroc- and 10 placebo-treated patients. In the cenicriviroc and placebo arms, 4 and 3 patients showed improvement of fibrosis in the year 1 biopsy, respectively (Figure 6A). Despite the small sample size not intended to assess FPS, post-treatment FPS modulation measured by CES showed a trend of association with the improved fibrosis in the cenicriviroc arm, while it was not obvious in the placebo arm (Figure 6B, C). These results may suggest that FPS modulation is more strongly correlated with pharmacological fibrosis improvement compared to spontaneous change, especially in such short timeframe (1 year). Of note, the proportion of patients with substantial CES reduction (i.e., FPS response) was comparable between the CENTAUR trial (22%) and the ex vivo PCLS tissue culture (31%) (see Figure 5A), suggesting the potential clinical utility of the short-term ex vivo PCLS culture to predict clinical anti-fibrotic responses to the therapy.

In the cenicriviroc arm, the FPS genes were suppressed or unchanged in patients who showed histological fibrosis improvement, whereas the genes were generally induced in patients with no fibrosis improvement (Figure 6D). A comprehensive assessment of molecular pathway modulation in global hepatic transcriptome revealed that the fibrosis responders showed suppression of specific fibrogenic pathways, which was not observed in the non-responders (Figure 6E, Supplementary table 10). The E2F signaling, previously implicated in chemically-or physiologically-induced liver fibrosis in mice,[38] was most strikingly suppressed, followed by the Wnt/β-catenin signaling only in the responders.

Interestingly, other well-known fibrogenic pathways, i.e., TGF-β and platelet derived growth factor receptor β (PDGFRB) pathways were unchanged, suggesting that these pathways are irrelevant to anti-fibrotic effect of cenicriviroc. This finding suggests that the E2F pathway may be an indicator of cenicriviroc response and represent a target to address absence of anti-fibrotic response.

Nuclear receptor signaling pathways such as peroxisome proliferator-activated receptor (PPAR), retinoid X receptor (RXR), retinoic acid receptor (RAR), and farnesoid X receptor (FXR) have been explored as therapeutic targets in NASH.[8] In recent clinical trials, combination therapies that involve agonists of the pathways have been actively evaluated to achieve clinically meaningful anti-fibrotic effect in NASH patients.[39] Of note, the fibrosis responders are characterized by enrichment of PPAR pathways after the 1-year cenicriviroc treatment, whereas the non-responders lack such modulation of the pathway (Figure 6F). There was no obvious induction of nor difference in RXR, RAR, and FXR pathways in both responders and non-responders. Assessment of experimentally-defined transcriptional target gene signatures of PPARα, δ, and γ agonists revealed that PPARα is dominantly induced by cenicriviroc in mouse primary hepatocyte (see Supplementary table 10). Induction of pharmacological PPARα target genes in the fibrosis responders was also confirmed in human primary hepatocytes.[40] Combination of cenicriviroc and a PPARα agonist, bezafibrate, led to reduced expression of genes encoding extracellular matrix proteins in a patient-derived liver spheroid (Figure 6G). These findings suggest that combination with PPARα agonists may improve the anti-fibrotic efficacy of cenicriviroc in non-responders, which warrants further assessment in future studies.

Serum-based FPS for non-invasive assessment of fibrosis progression risk

Despite the encouraging prognostic capability of FPS, requirement of liver biopsy tissue will limit its clinical applicability. We recently demonstrated that tissue transcriptome signature can be translated into serum-protein-based surrogate biomarker by utilizing our in silico pipeline, TexSEC (www.tex-sec.app).[41] By utilizing the established pipeline, we defined a 7-protein FPS surrogate, Fibrosis Progression Secretome signature (FPSec), which showed significant correlation with the FPS gene expression (*FDR* <.001) (Supplementary table 11). FPSec was tested using archived serum samples from a cohort of 79 Japanese cirrhosis patients with mixed etiologies reported in our previous studies. As expected, we observed significant concordance between the tissue-mRNA- and serum-protein-based prognostic risk prediction ($p<0.001$, Fisher's exact test) (Figure 7A). We further tested if the FPSec predicts development of hepatic decompensation as a measure of fibrotic disease progression and a surrogate of fibrosis progression in 122 patients with compensated cirrhosis from mixed etiologies analyzed in our previous study[7] (FPSec validation set) (Supplementary table 1). During a median follow-up of 5.5 years (IQR, 1.8 - 12.1 years), 29 patients developed hepatic decompensation. High-risk FPSec (n=62, 51%) was significantly associated with incidence of hepatic decompensation (hazard ratio [HR], 3.94; 95% CI, 1.59 - 9.78), which is superior to the prognostic association of PLSec reported in our previous study (HR, 3.51; 95% CI, 1.61 - 7.63)[7] (Figure 7B), and remained significant even after adjustment for a clinically available score, ALBI-FIB-4 score,[42] (adjusted HR, 3.00; 95% CI, 1.16 – 7.79) (Supplementary table 4). FPSec showed superior goodness of fit compared to ALBI-FIB-4

score (likelihood ratio test $P < .001$), and improved goodness of fit of ALBI-FIB-4 alone (likelihood ratio test $P = .02$). These results warrant further validation of FPSec as a non-invasive biomarker to assess risk of future fibrosis progression. In addition, given that the FPS-based risk status could change overtime spontaneously or in response to lifestyle or therapeutic interventions (Supplementary figure 4), this blood-based assay will enable more detailed time-series analysis to gain insight about how the molecular risk of fibrosis progression evolves over the natural history of chronic liver diseases.

DISCUSSION

The lengthy process of liver fibrosis progression has hampered discovery and validation of biomarkers predictive of long-term fibrosis progression. To overcome the change, we utilized patient cohorts with naturally-occurring (i.e., HIV infection) and iatrogenic (i.e., immunosuppressant use post transplantation) immune-suppressive conditions that accelerate fibrosis progression in the discovery of the FPS. The significant prognostic association for both PLS and FPS supports their utility as surrogate biomarkers to reliably estimate future fibrosis progression from the earliest stages with no to minimal fibrous tissue across patients, representing the major liver disease etiologies, i.e., chronic HCV infection and NAFLD. The clinical impact of such biomarkers to identify a subset of patients with rapid disease progression cannot be overemphasized, given the vast size of the population with early-stage chronic liver disease, the majority of which will be indolent. Our FPS can help optimize the allocation of limited medical resources to the at-risk patients.

Our recent study demonstrated that serum-based PLS can monitor dynamic change of prognostic risk level over the course of antiviral treatment in patients with chronic hepatitis C and this change is correlated with future disease progression.[11] This suggests that the signature can be used as a surrogate endpoint in clinical trials of anti-fibrotic agents to estimate their long-term prognostic impact within the typical timeframe of clinical trial and study (e.g., 5 years). In addition, a high-risk FPS may be used as a selection biomarker to indicate anti-fibrotic therapies and/or to guide patient enrollment in anti-fibrotic clinical trials. Our current study demonstrated that similar therapeutic modulation of FPS can be monitored even in the short-term ex vivo treatment of clinical PCLS. This encouraging finding indicates that rapid ex vivo assessment may serve as “avatar” for each individual patient to predict anticipated therapeutic benefit prior to initiation of the therapy. Furthermore, ex vivo testing in a cohort of patients enables exploration of response-associated clinical factors, which may guide study design of subsequent clinical trials. Collectively, our FPS should therefore facilitate clinical testing of experimental anti-fibrotic agents.

The FPS also provides clues to genetic drivers of fibrosis progression/resolution as targets for new anti-fibrotic strategies and/or to resolve resistance to existing therapies. The confirmed prognostic association in multiple clinical cohorts would support confidence in their clinical relevance. Genetic targeting has been increasingly recognized as a clinically viable therapeutic option with the recent FDA approval of oligonucleotide-based, liver-directed therapy.[43] Hepatic-cell-type-specific delivery of gene-targeting reagents is now also feasible.[44] Our gene-signature-based integrative systems biology approach also identified

small molecular compounds that mimic genetic targeting toward the FPS member genes. Furthermore, the characterization of genetic targets specific to each compound enables systematic identification of rational combination anti-fibrotic therapies as demonstrated by the example of EGCG-based combinations with BCL2-targeting compounds. It may help maximize anti-fibrotic efficacy, while mitigating toxicity by reduced dosing for each agent in the combinations. It is intriguing that the E2F pathway may be the major anti-fibrotic target of cenicriviroc, whereas a recent preclinical study reported that loss of E2F1 in hepatocytes promotes cholesterol accumulation and hepatic fibrogenesis in high-cholesterol-diet-fed mice.[45] Future studies should seek to reconcile the findings.

We note several limitations of the study. First, despite the successful validation for fibrosis progression, it is yet to be validated for association with longer and more definitive outcomes such as overall and/or liver-related death. Second, other major etiologies of fibrotic liver disease such as HBV infection and alcohol abuse need to be assessed in future studies. Lastly, the serum-based surrogate of FPS, FPSec, still awaits validation for fibrosis progression. Nevertheless, its capability to assay serum samples will enable more flexible testing for expanded clinical scenarios such as longitudinal repeated measurements.

Our study showcases a new strategy of prognostic-risk-based individualized patient management and biomarker-guided anti-fibrotic drug development to facilitate clinical translation of promising experimental anti-fibrotic agents. We anticipate that our integrative strategy will contribute to transformative improvement of the dismal prognosis of the patients with chronic fibrotic liver diseases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Conflict of interest

This study is partially supported by research fund from AbbVie (for the ex vivo tissue culture with cenicriviroc, hepatic transcriptome profiling of NASH patients enrolled in the CENTAUR trial), which did not have any role in the collection, analysis, and interpretation of data. YH is an advisor of Helio Health. YH and TFB as inventors of an international patent WO 2016174130 A1. TFB is a founder, shareholder, and consultant and YH is a shareholder of Alentis Therapeutics.

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Background and Context

Biomarkers to monitor trajectory of liver fibrosis progression are needed to predict prognosis and gauge clinically-meaningful benefit of new anti-fibrotic therapies in chronic liver disease patients.

New Findings

We developed liver-tissue- and serum-based Fibrosis Progression Signature (FPS), predicting long-term fibrosis progression in viral and metabolic liver disease patients and monitoring response to anti-fibrotic agents in experimental systems.

Limitations

The prognostic association should be externally validated in prospective cohort studies and clinical trials.

Impact

The FPS assays will enable personalized management of chronic liver disease patients according to individual prognostic risk and facilitate development of anti-fibrotic drugs with prognostic benefit.

Lay summary

Liver- and serum-based Fibrosis Progression Signature (FPS) assays were developed to predict future liver fibrosis progression and monitor response to anti-fibrotic therapies in chronic liver disease patients.

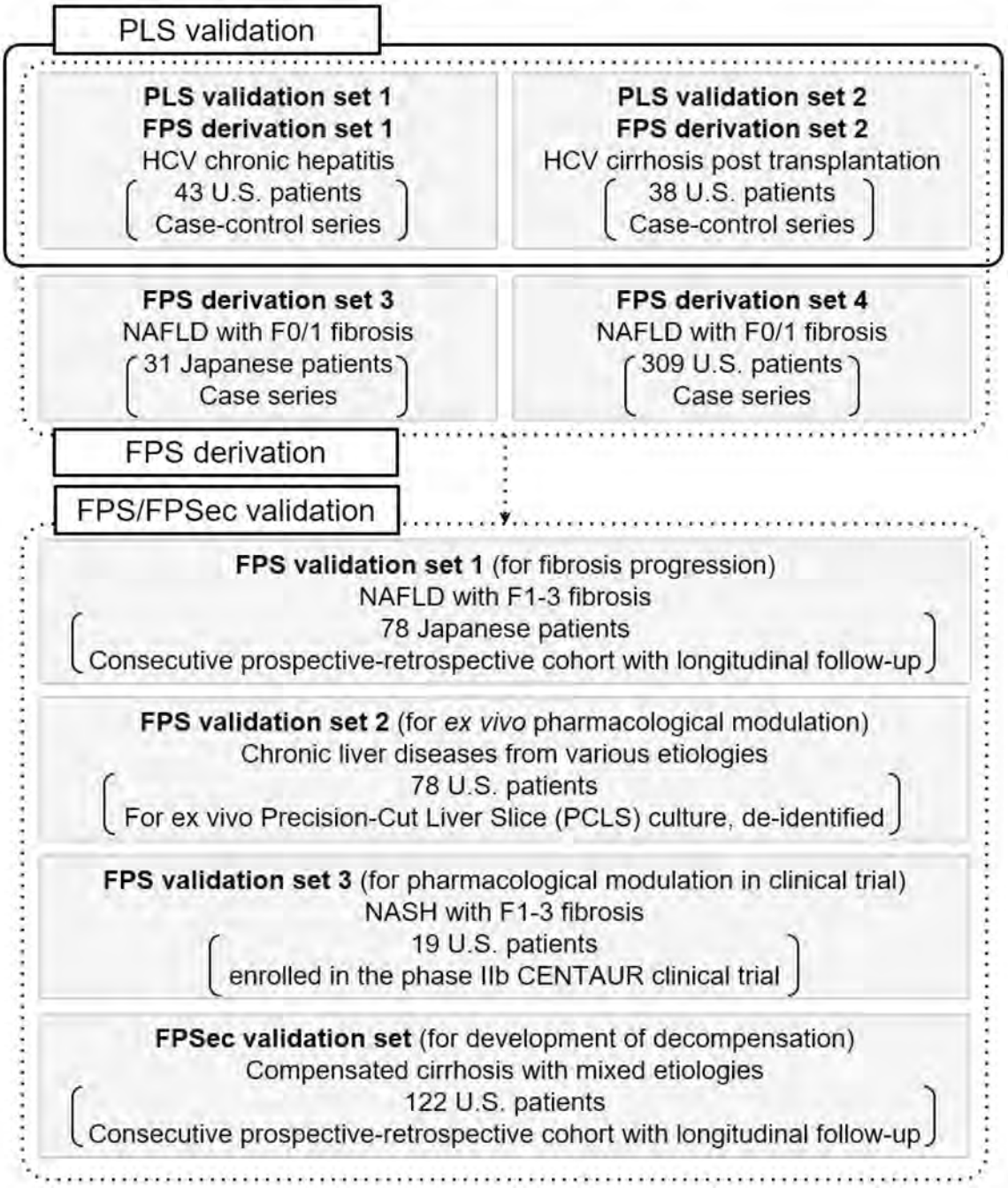

Figure 1. Study design.

Study design of Prognostic Liver Signature (PLS) validation and Fibrosis Progression Signature (FPS) derivation and validation.

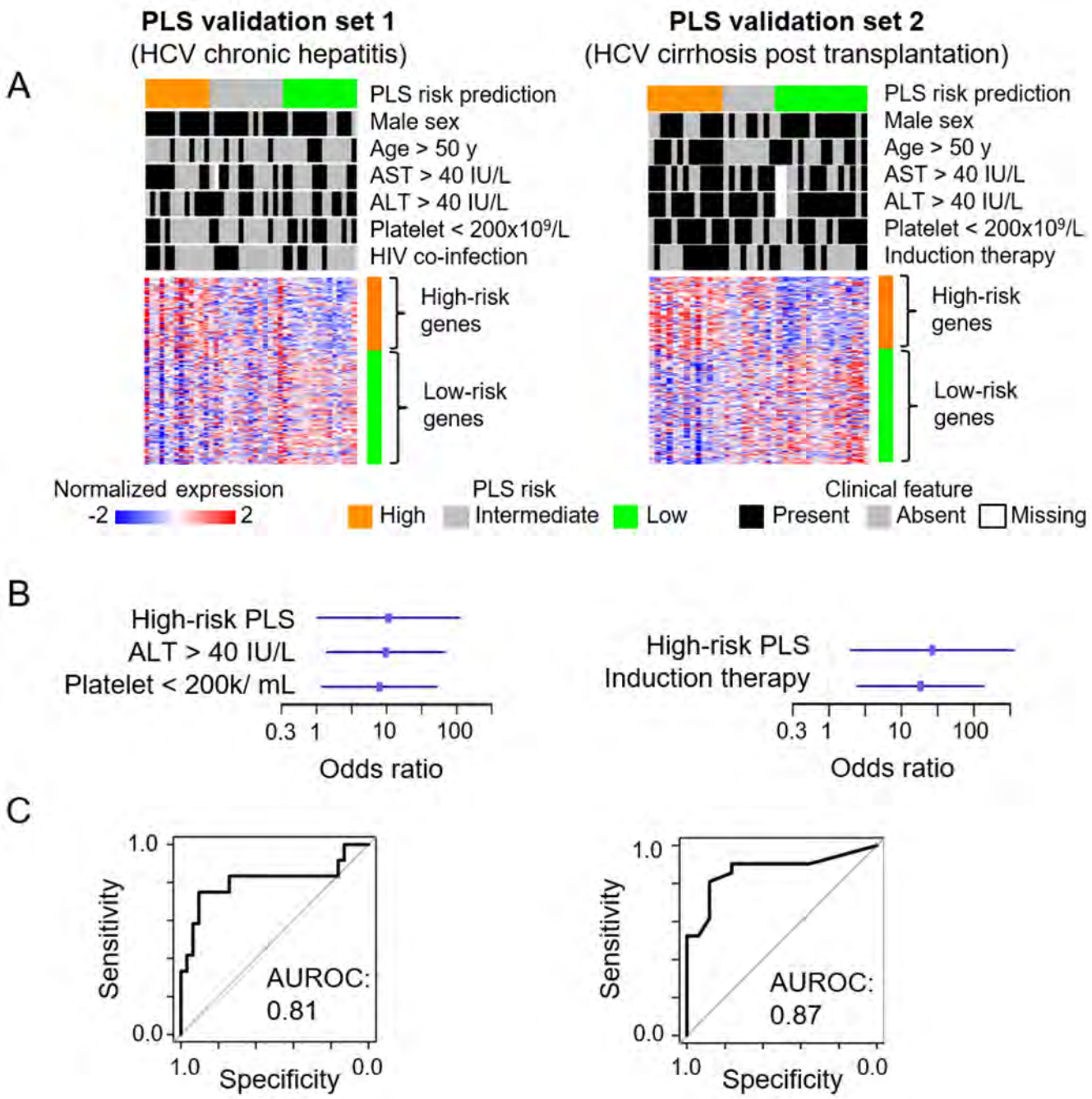

Figure 2. Validation of Prognostic Liver Signature (PLS) for 5-year fibrosis progression.

(**A**) Expression pattern of the PLS genes, (**B**) Odds ratios (blue squares) and 95% CI (horizontal line) for high-risk PLS and clinical prognostic variables in multivariable logistic regression, and (**C**) AUROC curve of the PLS-based prognostic prediction for 5-year fibrosis progression in the PLS validation set 1 (left) and 2 (right).

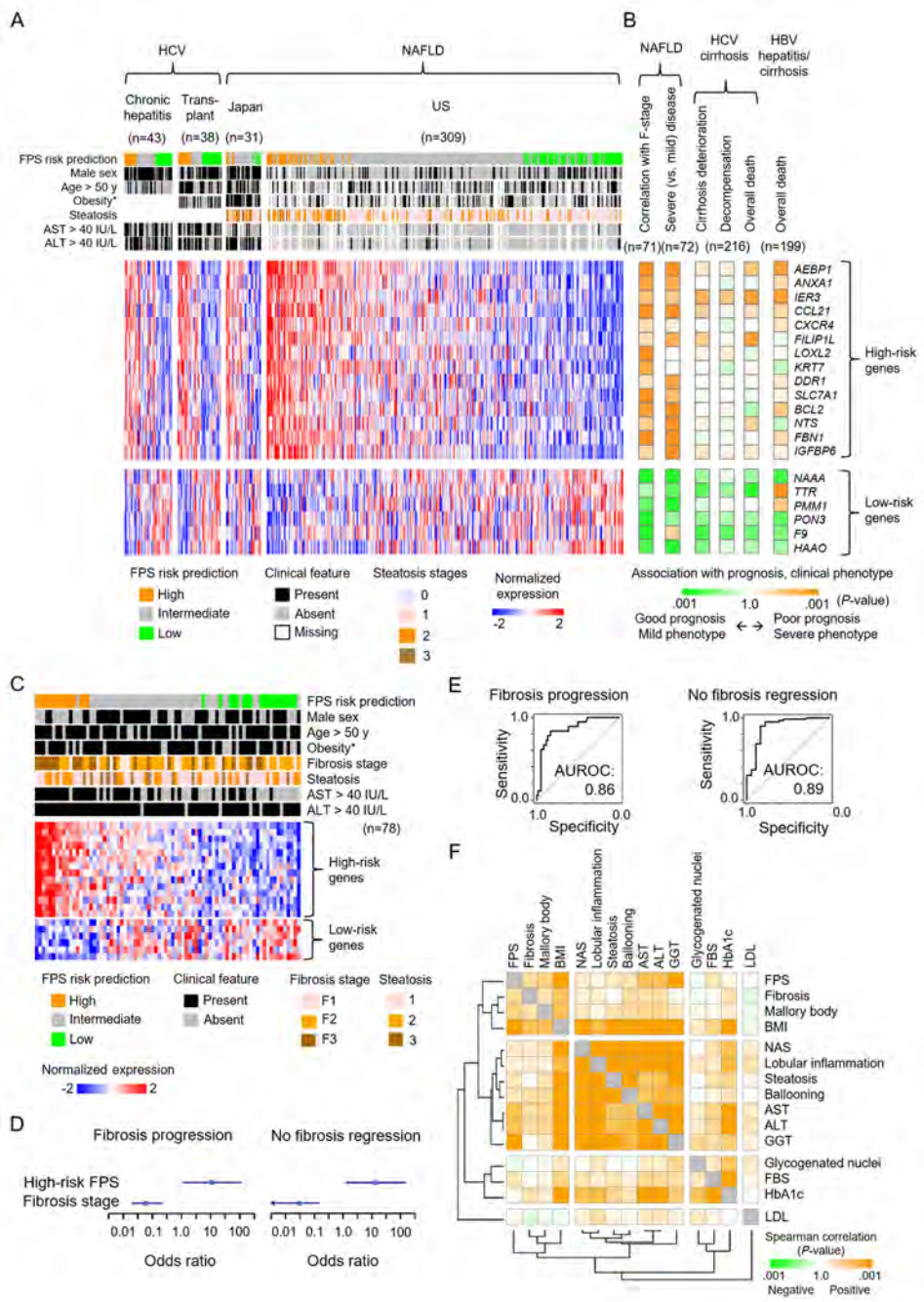

Figure 3. Derivation and validation of Fibrosis Progression Signature (FPS).

(**A**) Expression pattern of the FPS genes in the FPS derivation sets 1 to 4. (**B**) Association of each FPS member gene with NAFLD fibrosis stage (n=71) and histological severity (n=72; “severe” and “mild” indicate F3-4 and F0-1 fibrosis, respectively), HCV cirrhosis (n=216) and HBV chronic hepatitis/cirrhosis (n=199) prognosis (see Supplementary table 7). (**C**) Expression pattern of FPS and clinical annotations in the FPS validation set 1 (n=78). (**D**) Odds ratios (blue squares) and 95% CI (horizontal line) for high-risk FPS and clinical prognostic variable in multivariable logistic regression, and (**E**) AUROC of the

FPS-based prognostic prediction for fibrosis progression (left) and no fibrosis regression (right). (**F**) Correlation of the time-interval-adjusted change in FPS-based prognostic risk level (measured by combined enrichment score [CES]) with the changes in histological, biochemical, and clinical variables between the two time points of liver biopsy. *Obesity is defined by the WHO guidelines (i.e., BMI > 30 kg/m^2)[46] for the U.S. cohorts and the Asian-Pacific guidelines (i.e., BMI > 25 kg/m^2)[47] for the Japanese cohorts, considering race/ethnicity-specific impact of BMI on metabolic disease and prognosis.

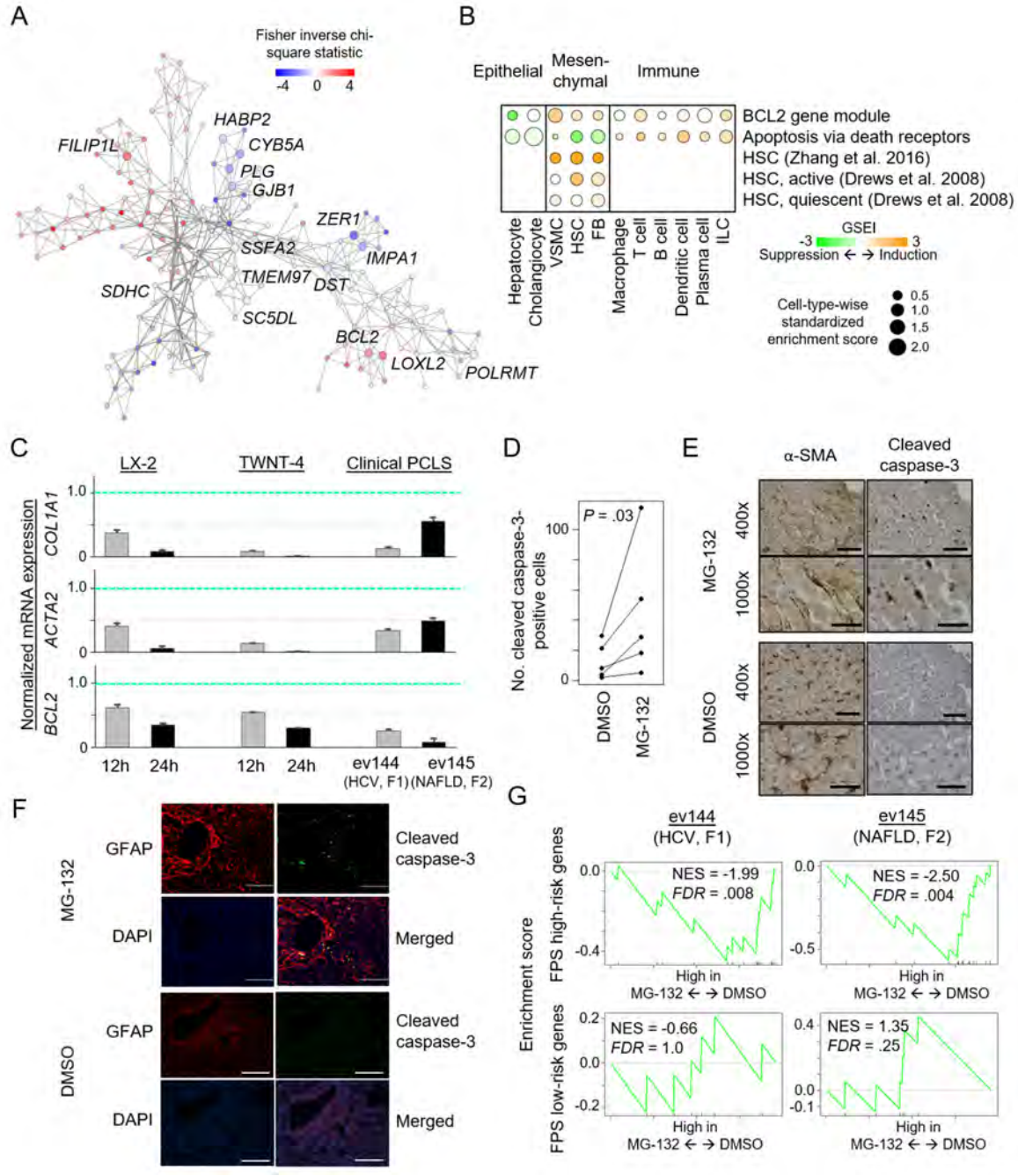

Figure 4. BCL2 is an FPS-associated anti-fibrosis target in clinical fibrotic liver tissues.

(**A**) Co-expression gene network defined in the FPS derivation set 1 to 4. Hub genes are indicated with larger nodes. Synthesized association with time to fibrosis progression (Fisher's inverse chi-square statistic) in the FPS derivation set 1 and 2 is shown by red (poor outcome) to blue (good outcome) color scale. (**B**) Dysregulation of BCL2-co-expressed gene module, apoptosis-related gene set, and hepatic stellate cell (HSC)-related gene signatures (see Supplementary methods – "Modulation of gene signatures and molecular pathways" section for specific citation information) in each cell type in single-cell RNA-Seq of human

cirrhotic livers. (**C**) Reduced expression of *COL1A1*, *ACTA2* (encoding α-smooth muscle actin [SMA]), and *BCL2* with MG-132 in LX-2 and TWNT-4 cells, and organotypic ex vivo culture of clinical fibrotic precision-cut liver slice (PCLS) tissues from 2 patients (ev144 [HCV, F1], ev145 [NAFLD, F2]). All assays were performed in triplicates. Green dotted line indicates expression level of the DMSO-treated control. (**D**) Difference in number of cells positive for cleaved caspase-3 per unit area between replicated PCLS tissues cultured with MG-132 or DMSO from five patients. Paired tissues from the same patient are connected with a line. Wilcoxon signed-rank test p-value is shown. (**E**) Immunohistochemical staining of α-SMA and cleaved caspase-3 in MG-132-treated (upper panel) and DMSO-treated (lower panel) clinical PCLS tissue (ev145). Scale bars indicate 50 μm and 25 μm for upper and lower panels, respectively. (**F**) Immunofluorescence staining of an HSC marker, glial fibrillary acidic protein (GFAP) (red), and cleaved caspase-3 (green) showing their co-localization (yellow) in MG-132-treated (upper panel) and DMSO-treated (lower panel) clinical PCLS tissue (ev145). Scale bars indicate 100 μm. (**G**) Modulation of FPS high- and low-risk genes measured by gene set enrichment analysis in the clinical PCLS tissues. NES: normalized enrichment score. FDR: false discovery rate.

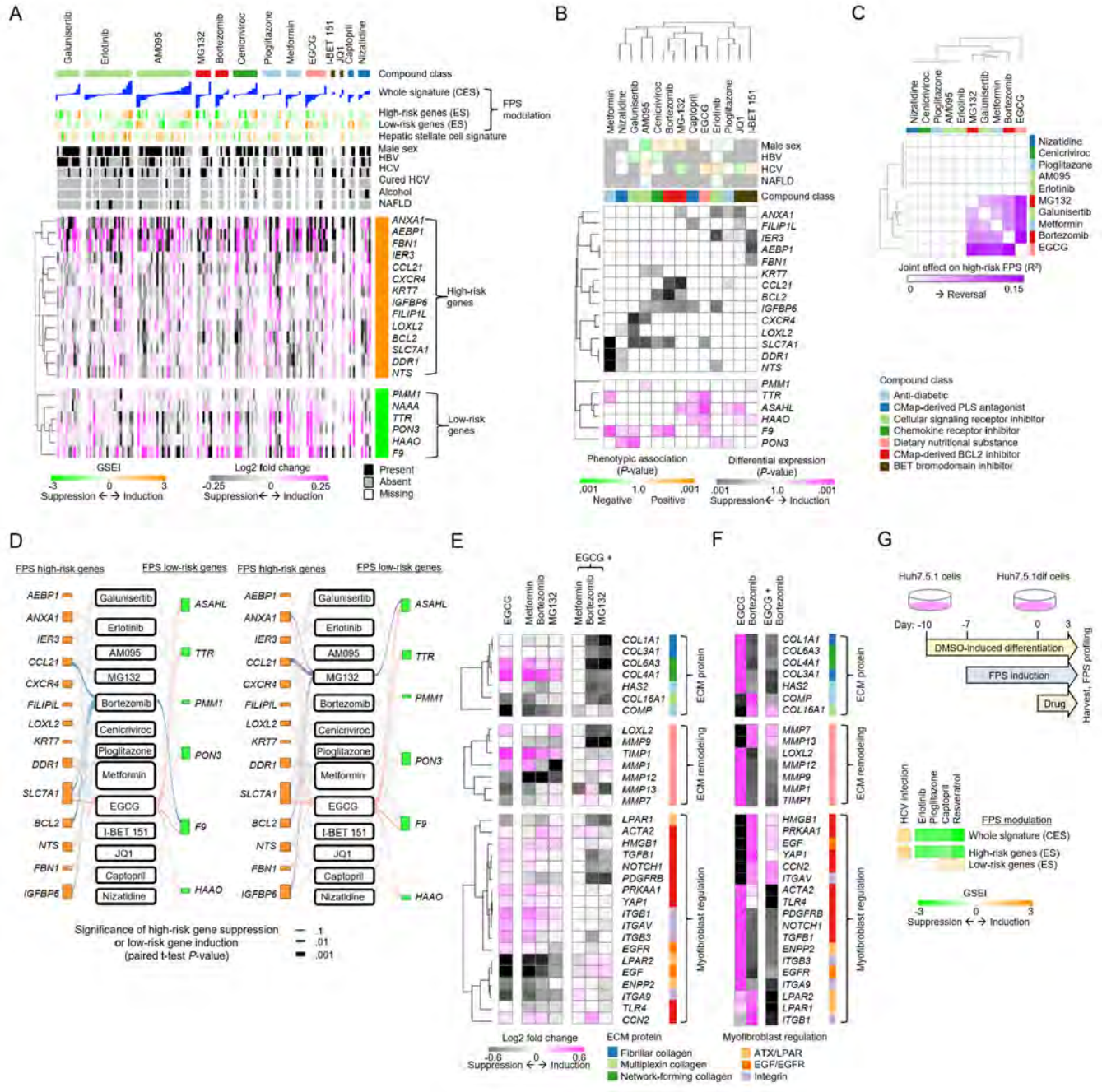

Figure 5. FPS-based systematic evaluation of anti-fibrotic agents in ex vivo culture of clinical PCLS tissues.

(**A**) Patient-level modulation of FPS genes by a panel of anti-fibrotic agents in organotypic ex vivo culture of PCLS tissues in FPS validation set 2 (Supplementary table 1). Patients are ordered by favorable modulation of FPS measured by CES from left to right. (**B**) Drug-level modulation of FPS to depict shared and unique target FPS genes across the tested anti-fibrotic agents. Phenotypic association of CES and differential gene expression were tested by Wilcoxon rank-sum test (when ≥ 2 samples were available in each group) and paired t-test, respectively. (**C**) Computationally inferred joint effect of combination of the

tested anti-fibrotic agents. (**D**) Complementary targeting of FPS genes by combining EGCG with bortezomib (left) or MG-132 (right). (**E**) Validation of the inferred joint effect of the combination therapies profiled by the liver fibrosis gene panel in *ex vivo* culture of a clinical fibrotic PCLS tissue. (**F**) Validation of the inferred joint effect of the combination therapies profiled by the liver fibrosis gene panel in *in vitro* culture of a patient-derived liver spheroid. (**G**) *In vitro* pharmacological FPS modulation in a cell culture system.

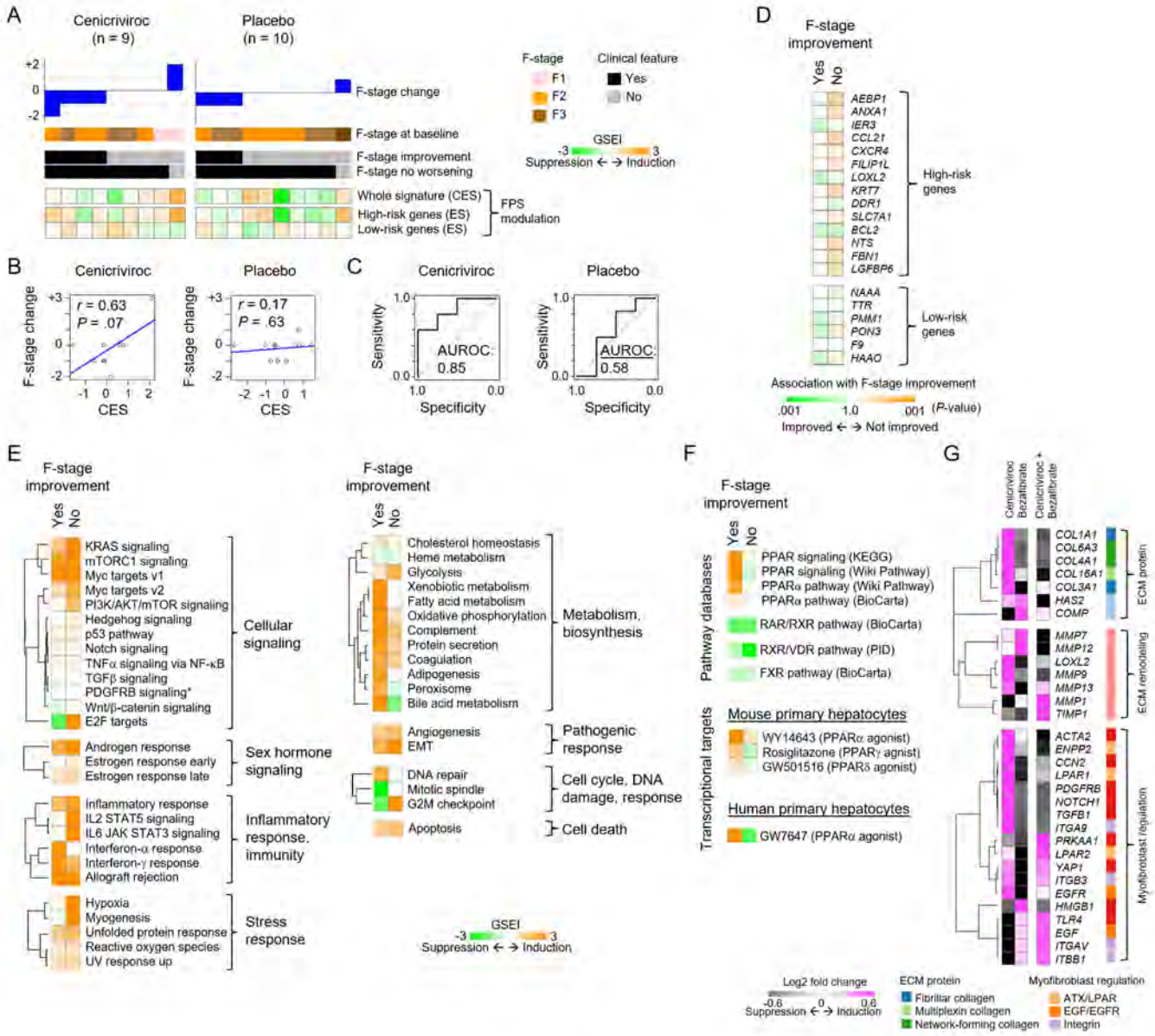

Figure 6. Modulation of FPS and molecular pathways by cenicriviroc in a phase II clinical trial.

(**A**) F-stage change and FPS modulation in cenicriviroc- and placebo-treated NASH patients. (**B**) Correlation between FPS modulation measured by CES and F-stage change. (**C**) AUROC for association between CES and 1-year histological fibrosis change. Modulation of (**D**) FPS member genes, (**E**) molecular pathways, and (**F**) nuclear receptor signaling pathways with the 1-year cenicriviroc treatment in patients with (yes) or without (no) F-stage improvement. GSEI: gene set enrichment index. (**G**) Validation of the inferred joint effect of the combination therapies profiled by the liver fibrosis gene panel in *in vitro* culture of a patient-derived liver spheroid.

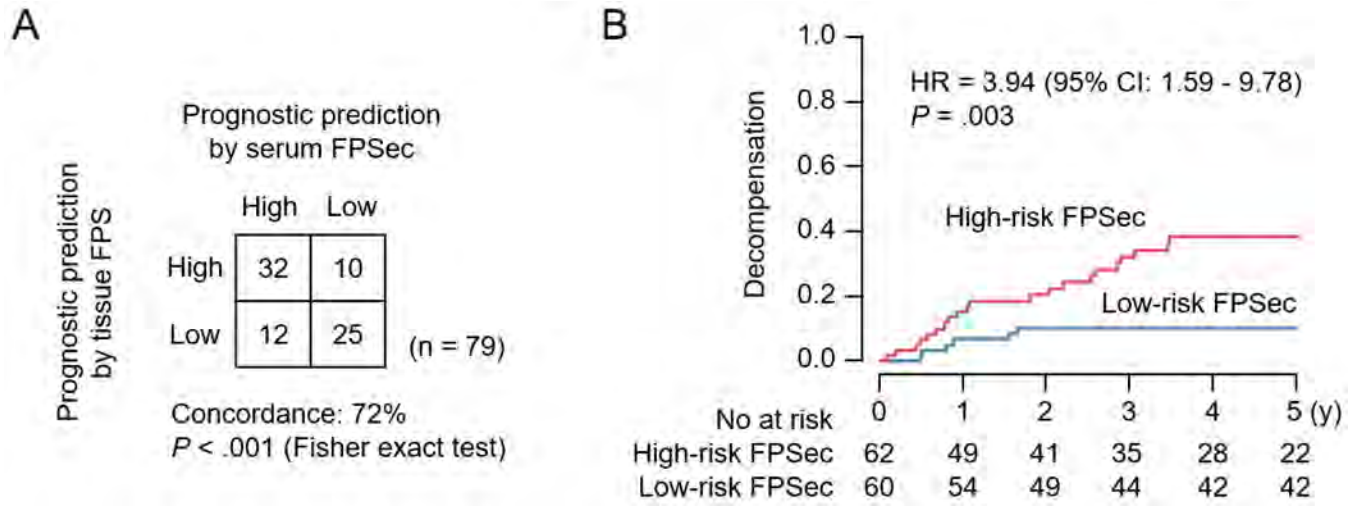

Figure 7. Derivation and validation of Fibrosis Progression Secretome signature (FPSec).

(**A**) Correlation of prognostic prediction between tissue-transcriptome-based FPS and serum-protein-based FPSec in a cohort of Japanese cirrhosis patients with mixed etiologies (n=79) from our previous publication.[7] (**B**) Validation of the FPSec in an independent cohort of American patients with compensated cirrhosis (n=122) for development of incident hepatic decompensation (the hazard proportionality test $P = .17$).