Molecular liver cancer prevention in cirrhosis by organ transcriptome analysis and lysophosphatidic acid pathway inhibition


1Division of Liver Diseases, Department of Medicine, Liver Cancer Program, Tisch Cancer Institute, Graduate School of Biomedical Sciences, Icahn School of Medicine at Mount Sinai, New York, U.S. 2Department of Gastroenterological Surgery, Graduate School of Medical Sciences, Kumamoto University, Kumamoto, Japan 3Division of Surgical Oncology, Massachusetts General Hospital

Correspondence: yujin.hoshida@mssm.edu, BFUCHS@mgh.harvard.edu.

Co-first author

Lead Contact

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All genomic datasets used in this study are available at NCBI Gene Expression Omnibus (www.ncbi.nlm.nih.gov/geo, accession number GSE64520).

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S.N., L.W., B.C.F., and Y.H. designed, planned and performed experiments and analyzed the results.
W.S., X.S., B.Z., and Y.H. defined and analyzed the liver cirrhosis gene modules.
Y.L. and S.L.F. provided transcriptome data of fibrosis mouse models.
G.B. provided AM063 and AM095.
T.H., S.G., R.S.K., C.B.B., and D.J.E. performed in vitro shRNA and compound treatment experiments.
M.M. and V.N. performed genomic profiling assays.
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B.C.F. and Y.H. conceptualized and supervised the project, analyzed results and wrote the manuscript.
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Hospital Cancer Center, Harvard Medical School, Boston, U.S. 4Department of Genetics and Genomic Sciences, Icahn School of Medicine at Mount Sinai, New York, U.S. 5Division of Gastroenterology and Hepatology, Geneva University Hospital, Geneva, Switzerland 6Institut National de la Santé et de la Recherche Médicale, U1110, Institut de Recherche sur les Maladies Virales et Hépatiques, Université de Strasbourg, Strasbourg, France 7Pharmakea Therapeutics, San Diego, U.S. 8Department of Pathology, Massachusetts General Hospital and Harvard Medical School, Boston, US 9Recanati/Miller Transplantation Institute, Icahn School of Medicine at Mount Sinai, New York, U.S. 10Hepatobiliary Division, Department of Internal Medicine and Hepatitis Center, Kaohsiung Medical University Hospital, and Faculty of Medicine, College of Medicine, and Center for Infectious Disease and Cancer Research, Kaohsiung Medical University, Kaohsiung, Taiwan 11Institute of Biomedical Sciences, National Sun Yat-Sen University, Kaohsiung, Taiwan 12Department of Hepatology, Toranomon Hospital, Tokyo, Japan 13Department of Neurology, Icahn School of Medicine at Mount Sinai, New York, U.S. 14Division of Thoracic Surgery, Massachusetts General Hospital Cancer Center, Harvard Medical School, Boston, U.S. 15M. & A. Migliavacca Center for Liver Disease and 1st Division of Gastroenterology Fondazione IRCCS Cà Granda Ospedale Maggiore Policlinico, University of Milan, Milan, Italy 16Liver Cancer Translational Research Laboratory, Barcelona Clinic Liver Cancer Group, Liver Unit, Institut d'Investigacions Biomèdiques August Pi i Sunyer (IDIBAPS), Hospital Clínic, CIBERehd, Universitat de Barcelona, Institució Catalana de Recerca i Estudis Avançats, Barcelona, Catalonia, Spain 17Cancer Program, Broad Institute of MIT and Harvard University, Cambridge, U.S. 18Pulmonary and Critical Care Unit, Center for Immunology and Inflammatory Diseases, Massachusetts General Hospital, Harvard Medical School, Boston, U.S. 19Institut Hospitalo-Universitaire, Pôle hépato-digestif, Nouvel Hôpital Civil, Strasbourg, France 20Liver Center, Gastrointestinal Division, Department of Medicine, Massachusetts General Hospital, Harvard Medical School, Boston, U.S.

SUMMARY

Cirrhosis is a milieu that develops hepatocellular carcinoma (HCC), the second most lethal cancer worldwide. HCC prediction and prevention in cirrhosis are key unmet medical needs. Here we have established an HCC risk gene signature applicable to all major HCC etiologies: hepatitis B/C, alcohol, and non-alcoholic steatohepatitis. A transcriptome meta-analysis of >500 human cirrhotics revealed global regulatory gene modules driving HCC risk and lysophosphatidic acid pathway as a central chemoprevention target. Pharmacological inhibition of the pathway in vivo reduced tumors and reversed the gene signature, which was verified in organotypic ex vivo culture of patient-derived fibrotic liver tissues. These results demonstrate the utility of clinical organ transcriptome to enable a strategy, reverse-engineering precision cancer prevention.

Graphical Abstract
INTRODUCTION

Prevention of disease progression has drastically improved patient mortality in several chronic diseases such as cerebro- and cardiovascular diseases, although cancer prevention has been a challenging task as evidenced by its almost unchanged mortality over the past several decades (Vogelstein et al., 2013). The major bottleneck remains the elusive mechanisms of carcinogenesis, and the difficulty in clinically verifying animal model-based findings due to the requirement for long-term monitoring of large number of cancer-free, asymptomatic individuals. In addition, the scarcity of cancer risk biomarkers hampers identification of high-risk populations that could be used to design clinical trials with practically feasible sample size and follow-up time (Hoshida et al., 2014). To overcome the obstacles for discovery of clinically relevant cancer chemoprevention targets and therapies, we have employed a “reverse engineering” approach, where cancer initiation-driving targets are first defined based on long-term clinical follow-up in multiple patient cohorts, and subsequently validated by experimental systems.

Liver cirrhosis is the terminal stage of chronic inflammatory and fibrotic liver diseases, and a distinct risk factor for developing hepatocellular carcinoma (HCC), the major histological type of liver cancer and the second leading cause of cancer mortality worldwide (Hoshida et al., 2014). Established cirrhosis is a strongly precancerous state with annual HCC incidence up to 8%, and complete removal of HCC tumors does not prevent subsequent, repeated de novo HCC development from remnant cirrhotic livers (70% recurrence rate within 5 years of surgical resection), resulting in incurable advanced-stage diseases and a persistently dismal prognosis (5-year survival rate generally less than 15%) (Forner et al., 2012). The strong carcinogenic “field effect” in the cirrhotic liver clearly indicates that cirrhosis is a rational target to explore cancer chemoprevention biomarkers and therapies (Hoshida et al., 2014). However, the diversity of etiological agents, namely hepatitis C virus (HCV), hepatitis B virus (HBV), alcohol abuse, and non-alcoholic fatty liver diseases (NAFLD)/non-alcoholic steatohepatitis (NASH), has precluded identification of broadly applicable cancer risk
biomarkers that could potentially salvage the HCC surveillance program that has collapsed due to the vast size of the cirrhotic population (Davila et al., 2010).

To address this unmet need and establish the first step of our “reverse-engineering” approach, we have identified and validated a 186-gene HCC risk-predictive signature in liver tissues (HCC risk gene signature), which was stable across multiple sampling sites in the liver and not affected by presence or absence of HCC tumor in the liver, in multiple independent patient cohorts enrolled from Asia, Europe, and the U.S., mainly affected by HCV infection and clinically followed for up to 23 years (Hoshida et al., 2008; Hoshida et al., 2013; King et al., 2015). The gene signature successfully monitored the HCC chemopreventive effect of an FDA-approved small molecule epidermal growth factor (EGF) pathway inhibitor, erlotinib, in multiple rodent models of cirrhosis-driven HCC (Fuchs et al., 2014), which led to initiation of a proof-of-concept, biomarker-guided cancer chemoprevention clinical trial (ClinicalTrials.gov, NCT02273362). However, it is still undetermined whether the gene signature is universally applicable beyond HCV. Moreover, adverse effects of erlotinib limit its wide-spread use as a preventive medicine and therefore indicate the necessity to further identify better HCC chemoprevention targets. Here our goals were to establish the clinical HCC risk gene signature assay in all major HCC etiologies, elucidate global molecular regulatory networks in cirrhotic liver for HCC chemoprevention target discovery, and to demonstrate the feasibility of fast-track, simultaneous identification of cancer chemoprevention targets, drugs, and biomarkers ready for clinical assessment.

RESULTS

Pan-etiology HCC risk gene signature

In our previous studies, the HCC risk gene signature has been validated for its association with future cancer risk in fibrotic/cirrhotic patients mainly affected by chronic HCV infection (Figure S1A, Cohort 1–4, n=668 in total) (Hoshida et al., 2008; Hoshida et al., 2013; King et al., 2015). Reanalysis of publicly available datasets of patient series affected with HBV, alcohol abuse, and NASH showed significant association of the signature with clinical outcome or disease severity (Figure S1B). To verify the findings with a clinically applicable assay, we implemented a reduced version of the 186-gene HCC risk gene signature bioinformatically defined in our recent study (32-gene signature) (King et al., 2015) in an FDA-approved digital transcript counting technology (Elements assay, NanoString). Technical validation demonstrated a high reproducibility of the gene expression measurements with the assay (Figure S1C). The assay was tested in liver tissues from an independent cohort of surgically-treated, early-stage HCC patients (n=263, Cohort 5) related to either of HCV, HBV, alcohol, or NAFLD/NASH, and followed for up to 23 years (Table S1). Complete removal of the tumors was histologically and radiologically confirmed. Recurrence hazard plot showed that the recurrences are dominantly de novo recurrence rather than dissemination/metastasis of the primary tumors (see Figure S1D for details). With the gene signature-based prediction, 57 (22%), 148 (56%), and 58 (22%) patients were classified into high-, intermediate-, and low-risk groups, respectively (Figure 1A). Concordant prognostic prediction between permutation of genome-wide and only signature genes was observed in our previously generated genome-wide transcriptome.
profiles (Cohort 1, Figure S1E). These risk groups were strikingly associated with de novo HCC recurrence (Figure 1B). Interestingly, the HCC risk gene signature quantitatively depicted the relative risk status of the liver according to etiology (Figure 1A). HBV-infected liver showed relatively lower risk status (p<0.001, Kolmogorov-Smirnov test) consistent with the clinical observation that HBV-related HCC is associated with less frequent postsurgical/ablative HCC development compared to HCV in both Western and Eastern patient cohorts (Franssen et al., 2014; Minami et al., 2015). In contrast, NAFLD/NASH-affected livers accumulated on the higher risk side (p<0.001) despite the lowest frequency of established cirrhosis (23% in NAFLD, 21% in NASH). This echoes the recently recognized epidemiological evidence that NAFLD/NASH-related HCC often develops in the liver with earlier-stage fibrosis (Kawada et al., 2009). Annual HCC incidence rate was nearly 4-fold higher in patients with high-risk prediction (41%/year) compared to low-risk prediction (11%/year) (Table S1). The prognostic association remained significant for each etiology subgroup in multivariable Cox regression adjusted for known clinical prognostic variables (Figure 1C). Of note, the prognostic association was significant in the subgroup of NASH patients (p=0.03), but not in NAFLD patients (p=0.14) in multivariable Cox regression. This indicates that presence of inflammation, i.e., steatohepatitis, is critical for the gene signature to be prognostic, and there is a need for complementary prognostic indicators for NAFLD patients who lack hepatic inflammation. Collectively, the results support panetiology prognostic capability of the gene signature assay in patients affected with HCV, HBV, alcohol, or NASH.

HCC risk prediction after pharmacological HCV eradication

The recent emergence of directly acting-antiviral regimens (DAAAs) for HCV has enabled high rates of complete viral eradication, i.e., sustained virologic response (SVR) (Chung and Baumert, 2014). Although SVR was epidemiologically associated with reduced HCC incidence in patients with advanced fibrosis, the risk of HCC is not eliminated and persists beyond a decade even after achieving an SVR (Morgan et al., 2013; van der Meer et al., 2012). Therefore, HCC risk prediction after SVR is urgently needed. Among the validation cohort (Cohort 5) patients infected with HCV (n=67), 4 patients achieved SVR prior to HCC development and showed relatively lower risk pattern of the HCC risk signature (Figure 1D), suggesting that SVR could modulate the molecular cancer risk status of the liver measured by the signature. Therefore, we next asked whether a change in the HCC risk gene signature expression after SVR is associated with future HCC risk by analyzing paired liver biopsy specimens obtained before and after antiviral therapy from 34 patients who never had HCC (median time from the second biopsy to the last observation: 6.0 years, IQR: 5.1–7.8 years). The magnitude of gene signature reversal, i.e., suppression of the HCC high-risk genes and restoration of the HCC low-risk genes in the signature, was the largest in 13 SVR subjects free of HCC at the end of follow-up (Figure 1E). Of note, 14 SVR patients who subsequently developed HCC showed less gene signature reversal even when compared to non-responders (NR) who did not develop HCC. Despite unsuccessful anti-HCV treatment, 4 out of the 5 HCC-free NR patients achieved resolution of clinically active hepatitis, i.e., normalization of elevated hepatic transaminases so-called biochemical response, which was associated with reduced HCC risk (Hoshida et al., 2014), consistent with the larger gene signature reversal. In contrast, the SVR patients who had HCC similarly achieved
normalization of transaminases, suggesting that the persisting HCC risk post SVR is not due to continued hepatic inflammation, but due to other unknown factors, which can be quantified by the HCC risk gene signature. These results collectively suggest that the gene signature assessment could be a promising quantitative HCC risk predictor after HCV eradication.

**Global transcriptomic landscape of fibrotic human livers**

We next explored pan-etiology targets of HCC chemoprevention by determining the systems-level dysregulation of the fibrotic/cirrhotic liver transcriptome. Regulatory gene network modeling was performed by synthesizing genome-wide transcriptome profiles of clinical fibrotic/cirrhotic liver tissues generated in our previous studies (Hoshida et al., 2008; Hoshida et al., 2013) (Cohort 1–3, n=523 in total, Figures 2A and S2A). Gene co-expression meta-analysis followed by Planar Filtered Network Analysis (PFNA) identified 31 tightly co-regulated gene modules, forming 2 major groups connected by 3 central hub modules (no.2, 3, and 8) (Figure 2B). Molecular pathways/functions, which have been implicated in the pathogenesis of liver inflammation, fibrogenesis, and carcinogenesis, were identified in the modules (Table S2). Gene module no.14, which was enriched with growth signaling pathways, contained the EGF pathway, which we identified as an HCC chemoprevention target in our previous study (Fuchs et al., 2014), which was directly connected to one of the central hub modules, suggesting a close regulatory relationship between them. Interestingly, the HCC risk gene signature was associated with known cancerous conditions in multiple organs such as colon mucosa affected with ulcerative colitis (but not Crohn’s disease, consistent with clinical observation), actinic keratosis (a precursor lesion of non-melanoma squamous cell carcinoma caused by long-term sun exposure), and diabetes (Calle et al., 2003; Itzkowitz et al., 2005) (Tables S3 and 4 and Figures S2B–E). Transforming growth factor-β (TGF-β), which was enriched in the stress response/RhoA module (no. 3), was inferred as a common top upstream regulator of the cross-organ carcinogenic gene dysregulations together with other cytokine-encoding genes, e.g., TNF and IL6 (Table S5), which indeed induced the HCC risk gene signature in lung epithelial cell line and organotypic ex vivo culture of non-diseased human liver tissues (Figures S2F and G). Indeed, RHOA is a transcriptional target of the TGF-β pathway (see “Transcriptional targets of TGF-β pathway activation” in Supplemental Experimental Procedures).

In human cirrhosis, the gene modules of stress response (no.2 and 3), extracellular matrix (ECM) (no.19), and interferon (no.24 and 28) were strongly induced, whereas the hepatocyte modules (no.9, 22, 23, and 26) were suppressed (Figure 3, Table S6). Several chemically, physiologically, or genetically-induced rodent models of liver fibrosis and/or HCC recapitulate the dysregulation of the HCC risk gene signature and the cirrhosis gene modules to varying degrees, underscoring their utility in testing chemoprevention strategies targeting specific gene module dysregulation. The repeated, low-dose diethylnitrosamine (DEN)-induced cirrhosis-driven HCC rat (low-dose DEN rat) is one of the models that showed the most striking induction of the HCC risk gene signature and global similarity in dysregulation of the cirrhosis gene modules. In this model, transcriptome profiles of hepatocyte and hepatic stellate cell fractions showed that the pattern of gene module dysregulation was generally comparable, suggesting the presence of intensive cross-talks between the cell
types, while induction of the growth signaling module and stress response/ECM modules was relatively stronger in hepatocytes and hepatic stellate cells, respectively. Therapeutic modulation, i.e., reversal of the dysregulated HCC risk gene signature and the cirrhosis gene modules, could also be monitored for anti-fibrotic and/or HCC chemopreventive treatments, including a small molecule EGF receptor inhibitor erlotinib (Fuchs et al., 2014), epigallocatechin gallate (EGCG, a green tea polyphenol), ghrelin (a “hunger” hormone), and vitamin D, depicting the utility of monitoring the cirrhosis gene modules in assessing clinical relevance of experimental HCC chemoprevention therapies.

Computational identification of LPA pathway as a functional key regulator of cirrhosis-driven carcinogenesis

In human cirrhosis, the central hub module, no.8, which is located at the center of the global cirrhosis gene networks, was the only module uniquely activated in association with increased risk of future de novo HCC recurrence, but not with presence of cirrhosis (Figure 3, the second column compared to the first column from the left), which suggested its specificity to carcinogenesis and reasoned us to explore the drivers of carcinogenesis in the module. In fact, computationally inferred hub key driver genes in the module and tightly-connected neighboring modules, no.2 and 3, were enriched with genes implicated in carcinogenesis: HINT1 has been described as a tumor suppressor gene in HCC (Calvisi et al., 2009; Zhang et al., 2009), UBE2K encodes an E2 ubiquitin ligase involved in p53 degradation (Saville et al., 2004), YY1 encodes an oncogenic transcription factor in HCC that is also known to suppress p53 pathway (Gordon et al., 2006), and ATP5J2, which encodes a protein known to physically interact with c-Myc (Figure 4A) (Agrawal et al., 2010). Indeed, HIP2 and YY1 expression was negatively correlated with p53 activation status, and ATP5J2 expression was positively correlated with induction of Myc pathway target genes in the 3 human fibrosis/cirrhosis cohorts (Cohort 1–3, Figures S3A–C). RHOA was another hub gene of the module no.3 together with its known target MRCL3, encoding myosin regulatory light chain, as well as fibrosis-related genes such as CCL5 (Berres et al., 2010) (Figure 4A).

To identify functional regulators of the genes in module no.8, we systematically surveyed enrichment of experimental genetic perturbation transcriptome signatures defined by shRNA library-based knockdown of 5,272 genes in an unbiased manner (the transcriptome signatures were generated using the dataset from NIH Library of Integrated Cellular Signatures [LINCS] project, www.lincsproject.org) (see Supplemental Experimental Procedures). Top enriched upstream regulator genes were AKT1, SLC35A1, DDX42, LPAR1, and ILK (Figure 4B). Indeed, Akt/mechanistic target of rapamycin (mTOR)/phosphoinositide 3-kinase (PI3K) pathway inhibitors, everolimus and sirolimus, are currently being tested in clinical trials for prevention of post-liver transplantation HCC recurrence (Burra and Rodriguez-Castro, 2015). Among the rest, LPAR1 was the only one encodes a protein selectively targetable with compounds currently under clinical development. Lysophosphatidic acid receptors (LPARs) form a family of G protein-coupled receptors, and LPAR1 has been shown to promote fibrosis in multiple organs by upregulating pro-fibrogenic mediators, including connective tissue growth factor (CTGF), known to be elevated in activated hepatic stellate cells (Huang and Brigstock, 2012; Pradere...
et al., 2007; Sakai et al., 2013; Tager et al., 2008). In liver, expression of autotaxin (ATX), which converts lysophosphatidylcholine (LPC) into LPA (ligand for LPAR), is elevated in serum of HCV-infected patients, especially in those with HCC and/or severe fibrosis (Cooper et al., 2007; Nakagawa et al., 2011; Watanabe et al., 2007). In consistent, AKAP13, an HCC risk signature member gene, encodes a guanine exchange factor that relays the signal to downstream of the RhoA signaling pathway, further supporting the role of LPA pathway as a functional driver of HCC development.

It is known that RhoA, Ras/mitogen-activated protein kinase kinase (MAPK)/extracellular signal–regulated kinase (ERK)/MAPK/ERK kinase (MEK), Akt/PI3K, and phospholipase C (PLC) pathways are downstream effectors of the LPA pathway (Kihara et al., 2015). In Cohort 5, expression of NCAPH (RhoA pathway target), PRKG2 (K-Ras pathway target), SERPINB2 (H-Ras pathway target), and GPX2 (MEK pathway target) gradually increased according to the HCC risk signature-based stratification (Figure S3D). Transcriptome-based unbiased in silico screening of 20,413 chemical perturbation in the LINCS database using the HCC risk gene signature as a query yielded selective inhibitors of Rho kinase, Akt/PI3K, and MAPK/ERK/MEK pathways, further supporting the involvement of the LPA downstream pathways (Table S7). These results collectively support that the LPA pathway activation is robustly observed in human and rodent cirrhotic livers at risk of HCC development, representing an HCC chemoprevention target.

In vivo pharmacological LPA pathway inhibition suppressed HCC development, and reversed HCC risk gene signature

We next examined HCC chemopreventive effects of LPA pathway inhibition by using selective ATX (AM063) and LPAR1 (AM095) inhibitors (Figure S4A). To examine involvement of the RhoA pathway, two Rho kinase (ROCK) inhibitors, Fasudil and Y-27632, were tested in a fibrogenic human hepatic stellate cell line, TWNT-4, along with AM095. All of the three compounds similarly suppressed LPA-induced phosphorylation of myosin regulatory light chain (pMRLC), indicator of RhoA pathway activation (Figure S4B). Suppression of pERK (downstream of another LPA target, MEK/Ras pathways), was unique to AM095, consistent with the established specificity of the compound to the LPA pathway (Stein et al., 2015; Swaney et al., 2011). In contrast, pAkt was suppressed by LPA pathway activation and restored by AM095, suggesting that the Akt and LPA pathways are in negative feedback with each other in the liver, and LPA signaling represents an HCC chemoprevention target distinct from the Akt pathway. TGF-β is one of the major profibrogenic factors in the liver, which has been extensively studied as a potential therapeutic target and is known as an upstream regulator of CTGF (Hoshida et al., 2014). TGF-β neutralizing antibody had no effect on the LPA downstream pathways. LPA-induced CTGF expression was suppressed by AM095 and, to a lesser extent, Y-27632 whereas TGF-β neutralizing antibody had no effect (Figure S4C). shRNA-based knockdown of LPAR1 and RHOA expression resulted in similar suppression of LPA-induced CTGF expression (Figures S4D and E).

In the low-dose DEN rat model of cirrhosis-driven HCC, which manifests global transcriptomic and histological similarity to human (Fuchs et al., 2014) (Figures 3 and S4F), 

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plasma ATX activity and hepatic Lpar1 expression gradually increased as cirrhosis developed (Figures 5A–5C). Hepatic Ctgf expression similarly increased mainly in hepatic stellate cell fraction (Figure S4G). Atx expression was higher in the hepatocyte fraction, whereas Lpar1 were expressed predominantly in the stellate cell fraction, collectively suggesting that cross-talk between the cell types results in the LPA pathway activation in the liver tissue (Figure S4H). Ten-week administration of AM063 or AM095 significantly reduced hepatic expression of Ctgf, collagen 1 (Colla1), and α-smooth muscle actin (α-SMA, Acta2), the major extracellular matrix proteins secreted from activated hepatic stellate cells (Figure S4I). Histological liver fibrosis was attenuated and, as bioinformatically predicted, HCC nodules were significantly reduced (Figures 5C–F). Correspondingly, transcriptome profiling of the rat livers by RNA-Seq demonstrated reversal of the HCC risk gene signature and dysregulation of human cirrhosis gene modules along with the LPA downstream pathways and the hepatic stellate cell gene signature (Zhang et al., 2016) (Figure 5G). The driver genes of the hub cirrhosis gene modules (no.2, 3, 8) and EGF pathway targets were suppressed by the compounds, whereas modulation of TGF-β pathway targets was moderate (Figure S4J). In summary, the pharmacological LPA pathway inhibition elicited HCC chemopreventive effect via suppression of RhoA and ERK pathways, but not Akt and TGF-β pathways. Neither of the compounds showed evidence of hepatotoxicity, while decreased serum levels of bilirubin was observed consistent with the histological improvement of liver fibrosis (Figure S4K).

HCC risk gene signature reversal by LPA pathway inhibition in organotypic ex vivo culture of human fibrotic liver tissues

Finally, we sought to determine whether the reversal of the HCC risk gene signature, which was accompanied with the in vivo HCC chemopreventive effect by LPA pathway inhibition, can be monitored in human liver tissues. We first confirmed that induction of the HCC risk gene signature as well as our hepatic stellate cell signature (Zhang et al., 2016) was preserved in organotypic ex vivo culture of carbon tetrachloride-induced fibrotic mouse liver tissue up to 48 hr, even without continued chemical treatment, suggesting that fibrotic tissue microenvironment is sufficient for perpetuation of the gene signature induction in the culture (Figure S5A). Subsequently, 13 clinical human liver tissues (male: 7, female: 6; HBV: 5, HCV: 3, NASH: 1, and cryptogenic: 4) were similarly cultured ex vivo with AM095 for 48 hr. HCC risk gene signature prediction was performed using the tissues before culture (Figures 6 and S5B). With the drug treatment, varying degrees of HCC high-risk gene suppression, HCC low-risk gene restoration, and/or combinatorial reversal were observed (Figure 6). The presence of HCC high-risk gene signature before the drug treatment (p=0.008, Wilcoxon rank-sum test) as well as less liver fibrosis (p=0.01, Pearson correlation test) were associated with greater gene signature reversal, suggesting that these factors may predict gene signature response to AM095 in actual clinical setting. At gene and molecular pathway levels, suppression of growth signaling (EGF, IGFBP6, EPHA4), extracellular matrix protein-encoding genes (COL6A3, COL16A1, LOXL2), cell adhesion and fibrogenic cytokines (LPP, PODXL, CTNND2, CXCR4, CCL21) and oxidative stress (GPX2, NOS2A) as well as restored expression of plasma protein-encoding genes (PLG, C8B, C5, TTR) were the main features observed in the gene signature responders. The hepatic stellate cell signature was suppressed in patients with reversed HCC risk gene signature (p=0.045,
Wilcoxon rank-sum test). These results suggest that the HCC risk gene signature or serum LPA level or proteomic surrogates of the tissue-based gene signature (Cooper et al., 2007; Muir et al., 2013; Nakagawa et al., 2011; Watanabe et al., 2007) could serve as a companion biomarker for pharmacological LPA pathway inhibition to identify potential responders and monitor the therapeutic effect in future HCC chemoprevention trials. In fact, when enrollment in adjuvant chemoprevention trials following curative treatment is limited to subjects with the HCC high-risk gene signature, the estimated sample size is comparable to those required in cancer therapeutic trials, i.e., up to a few hundred (Figure S5C).

DISCUSSION

Cancer chemoprevention is a major unmet medical need with numerous challenges during both preclinical and clinical development (Hoshida et al., 2014). It is typically not feasible to experimentally model the entire physiological process of cancer development in order to evaluate the effect of cancer-preventive intervention. Cancer chemoprevention clinical trials enrolling cancer-free asymptomatic individuals require larger sample sizes and longer follow-up time compared to advanced-stage cancer therapeutic trials due to a smaller event rate (Lippman et al., 2009). Even when clinical risk factors are known, thousands of individuals are generally required to be enrolled and followed for longer than 5 years to detect cancer preventive effect (Cuzick et al., 2014). Therefore, molecular biomarkers of cancer risk that further enrich high-risk population will lower the bar to conducting cancer chemoprevention trials. Our HCC risk signature, which was validated in all major HCC etiologies including post HCV cure, will have a wide range of applicability as a pan-etiology companion biomarker for HCC chemoprevention clinical trials.

The cirrhosis gene regulatory modules provide a systems-level landscape of molecular dysregulation common to a wide range of liver disease etiologies. Projection of the global transcriptome onto the cirrhosis gene module map will enable rapid and straightforward identification of experimental models that best resemble human cirrhosis according to the molecular targets of interest. Our study demonstrates that the LPA pathway is one of the central regulators of human cirrhosis pathogenesis and a promising target for HCC chemoprevention, one made more feasible by the existence of compounds in clinical development for non-cancer patients with negligible toxicity (Kihara et al., 2015). Although TGF-β pathway has been studied as an anti-fibrosis target, clinical inhibition of the pathway has been deemed challenging due to systemic toxicities and may not be justified as a preventive intervention for asymptomatic and still cancer-free individuals (Hoshida et al., 2014; Mehal and Schuppan, 2015). Our results suggest that LPA pathway inhibition is an alternative way to antagonize downstream targets to achieve HCC chemoprevention, while circumventing the toxicities of directly targeting TGF-β pathway.

Collectively, our study demonstrates that transcriptome analysis of cancer-prone, chronically-diseased organs with long-term clinical observation is a viable and effective approach to uncover cancer chemoprevention biomarkers and targets. This approach will be widely applicable to other cancer types driven by chronic organ inflammation and/or fibrosis, as a paradigm, reverse-engineering precision cancer prevention.
EXPERIMENTAL PROCEDURES

More detailed procedures are described in the Supplemental Information.

Patient cohorts

Prognostic association of the HCC risk gene signature was validated in an independent cohort of 263 consecutive patients with curatively resected HCC at Toranomon Hospital or Kumamoto University Hospital between 1988 and 2012. Paired liver biopsy tissues were obtained before and after anti-HCV therapy for patients with chronic hepatitis C at Toranomon Hospital and Kaohsiung Medical University Hospital between 2004 and 2009. The study was approved by institutional review board (IRB) at Mount Sinai Hospital, Toranomon Hospital, Kumamoto University Hospital, and Kaohsiung Medical University Hospital for anonymous analysis of de-identified archived waste FFPE tissues from previous routine clinical care without written informed consent.

HCC risk gene signature profiling

Archived FFPE liver tissues were analyzed by the 32-gene HCC risk signature (King et al., 2015) implemented in nCounter Elements assay (NanoString). Prognostic prediction was performed as previously described (King et al., 2015). Prognostic association of the gene signature was evaluated by log-rank test and multivariable Cox regression modeling adjusted for clinically well-established prognostic variables. All analyses were performed using R statistical language (www.r-project.org) and GenePattern (www.broadinstitute.org/genepattern).

Human cirrhosis regulatory gene modules

Functionally co-regulated gene modules in human fibrotic/cirrhotic liver tissues were determined in genome-wide transcriptome profiles of 3 independent patient cohorts (n=523) using Fisher’s inverse chi-square statistic (Fisher, 1932) and Planar Filtered Network Analysis (PFNA) algorithm (Song and Zhang, 2015). Induction or suppression of the gene modules as was assessed in a panel of in vitro and in vivo experimental models of liver diseases and HCC (Table S6) using Gene Set Enrichment Analysis (GSEA) (Subramanian et al., 2005).

In vitro and in vivo pharmacological LPA pathway inhibition

The ATX inhibitor AM063 and the LPAR1 antagonist AM095 were provided by Amira Pharmaceuticals and Bristol Myers Squibb, and tested in human hepatic stellate cell line, TWNT-4, and male Wistar rats (Charles River) treated with low-dose (50 mg/kg) weekly injection of diethylnitrosamine (DEN) as previously described (Fuchs et al., 2014) following Guide for the Care and Use of Laboratory Animals (National Academy of Sciences) and the institutional guidelines, and the protocol was approved by Massachusetts General Hospital Subcommittee on Research Animal Care. Tumor nodules were counted in serial liver sections, and primary hepatocytes and hepatic stellate cells were isolated from fresh liver tissue using an established protocol (Fuchs et al., 2014). Gene expression was measured by real-time PCR. Total/phosphorylated MRLC, collagen 1, and α-SMA protein levels were determined by western blotting as previously described (Fuchs et al., 2014). Liver fibrosis
stained by sirius red was quantified using Image J software (imagej.nih.gov/ij) as collagen proportionate area. Intergroup difference was evaluated by t-test with Bonferroni correction as needed.

**Organotypic ex vivo culture of experimental and clinical liver tissues**

Fresh liver tissues from carbon tetrachloride (CCl₄)-treated strain A/J male mice (Jackson Laboratory) and de-identified surgically resected human liver tissues (obtained via Mount Sinai Biorepository with IRB-approved written informed consent) were sliced into 300 µm-thick tissue sections, and the human livers were cultured with the LPA pathway inhibitor AM095 (3 µM) or DMSO for 48 hr. Expression of HCC risk gene signature was determined before and after the culture using the Elements assay, and modulation of the signature by AM095 was determined by GSEA.

**Transcriptome profiling of rodent livers**

Transcriptome profiling of rodent liver was performed by RNA-Seq, RatRef-12 beadarray, or MouseRef-8 beadarray (Illumina) as previously described (Fuchs et al., 2014). Rodent genes were converted into orthologous human genes using a mapping table obtained from NCBI HomoloGene database (build 68). Induction or suppression of target genes of relevant cellular signaling pathways was assessed by GSEA.

**Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.

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The HCC risk gene signature Elements assay probes are available upon request to the lead contact (Y.H.) or at www.hoshida-lab.org.

REFERENCES


SIGNIFICANCE

Chronically inflamed and/or fibrotic organ is a clinically well-known “soil” that develops cancer, although it has been challenging to identify clinically relevant cancer chemoprevention targets in the organ and therapies due to complex molecular dysregulation involving multiple cell types, lengthy process of carcinogenesis, and the lack of cancer risk biomarkers to enable their clinical testing. Here we show a strategy to systematically utilize diseased organ transcriptome to simultaneously identify clinically relevant cancer chemoprevention biomarkers, targets, and therapies by integrating clinical cancer risk based on several decades of clinical observation. This approach is applicable to other cancer types arisen from chronically diseased organs, and will facilitate development of molecular targeted cancer chemoprevention therapies.
HIGHLIGHTS

- Clinically applicable pan-etiology HCC risk biomarker was established.
- Global transcriptome map of cirrhosis identified HCC chemoprevention targets.
- Global transcriptome map of cirrhosis identified clinically-relevant animal models.
- LPA pathway inhibitors were verified as HCC chemopreventive and antifibrotic drugs.
Nakagawa et al. establish an hepatocellular carcinoma (HCC) risk gene signature applicable to all major HCC etiologies and identify the lysophosphatidic acid pathway as a central chemoprevention target, pharmacological inhibition of which reduces tumors and reverses the gene signature in preclinical models.
**Figure 1. HCC risk gene signature in the major HCC etiologies**

(A) Heatmap of the 32-gene HCC risk gene signature, which classified the patients (n=263) into high-, intermediate-, and low-risk groups as indicated as orange, gray, and green color bars, respectively. Black bars on top indicate presence of each HCC etiology.

(B) Probabilities of HCC (left) and overall survival (right) according to the gene signature-based HCC risk prediction. p values were calculated by log-rank test.

(C) Hazard ratios of HCC development according to HCC etiology in multivariable Cox regression modeling adjusted for clinically-established risk factors. Blue squares indicate hazard ratios, and horizontal bars indicate corresponding 95% confidence interval. BCLC stage, Barcelona Clinic Liver Cancer prognostic stage.

(D) Expression pattern of HCC risk gene signature in a subgroup of patients with HCV infection (n=67). Black bars indicate patients who achieved sustained virologic response (SVR) to anti-HCV therapy prior to HCC development.

(E) HCC risk gene signature in paired liver biopsies obtained before and after anti-HCV therapy in patients with chronic hepatitis C who achieved no response (NR) or SVR and subsequently developed HCC or remained HCC-free. Magnitude of signature change is presented as normalized enrichment score (NES) computed by Gene Set Enrichment Analysis. Values attached to each bar indicates false discovery rate (FDR).

See also Figure S1 and Table S1.
Figure 2. Human liver cirrhosis regulatory gene modules for discovery of HCC chemoprevention targets

(A) Transcriptomic meta-analysis of clinical liver fibrosis/cirrhosis cohorts to identify regulatory gene modules and putative key driver genes. Gene-gene correlation matrices in three human cirrhosis cohorts (left), synthesized gene-gene correlation matrix by using Fisher’s inverse chi-square statistic (middle), and workflow to identify regulatory gene modules and key driver genes (right) are shown. Rows and columns in the heatmaps represent genes in each cohort.
See also Figure S2 and Tables S2, S3, S4, and S5.
Figure 3. Cross-species/model comparison in the space of human cirrhosis regulatory gene modules

Activation status of each cirrhosis gene module was assessed by Gene Set Enrichment Analysis, and visualized as gene set enrichment index (GSEI) calculated from gene set enrichment p value based on iterative random gene permutations (1,000 times for each module in each condition). Orange and green colors in heatmap indicate statistical significance of induction and suppression of each gene module, respectively, in association with each phenotype or intervention in each model. GSEI of +3 indicates induction (orange) at enrichment p=0.001, GSEI of −3 indicates suppression (green) at enrichment p=0.001, and GSEI of 0 indicates no modulation (white) at enrichment p=1.0. In “Cirrhosis (human)” column, genes in the transcriptome dataset were rank-ordered according to differential expression between cirrhotic and healthy livers to compute GSEI. In “HCC high risk (human)” column, genes were rank-ordered according to association with time to HCC development measured by Cox score (Hoshida et al., 2008) to compute GSEI. DEN, diethylnitrosamine; CCl₄, carbon tetrachloride; BDL, bile duct ligation; MCD, methionine/
choline-deficient diet, HFD, high fat diet; HCD, high cholesterol diet; FLS, fatty liver Shionogi; HSC, hepatic stellate cell; LEC, liver endothelial cell; EGCG, epigallocatechin gallate.
See also Table S6
Figure 4. Bioinformatic identification of HCC chemoprevention targets

(A) Central hub gene modules no.2, no.3, and no.8 in human liver fibrosis/cirrhosis gene networks. Co-regulatory gene modules at the center of human liver fibrosis/cirrhosis regulatory gene networks determined by Planer Filtered Network Analysis (PFNA) in the genome-wide transcriptome profiles of 523 fibrotic/cirrhotic liver tissues (Cohort 1–3). Outline color of each node (gene) indicates gene module the gene belongs to (green, no.2; cyan, no.3; pink, no.8). Node color indicates correlation with HCC risk measured by Cox score in Cohort 1, from which the HCC risk gene signature was originally derived (red and...
blue colors indicate correlation with high and low HCC risk, respectively). Node size reflects connectivity to neighboring genes measured by degree. Putative key driver genes identified by Key Driver Analysis (KDA) are labeled with gene symbol.

(B) Functional regulators of the HCC risk-associated gene module (no.8). Enrichment of experimental genetic perturbation transcriptome signatures defined by shRNA library-based knockdown of 5,272 genes (down-regulated gene signatures by the gene knockdown) derived from NIH Library of Integrated Cellular Signatures (LINCS) project (www.lincsproject.org) in the HCC risk-associated gene module, no.8, was systematically assessed in an unbiased manner using Fisher’s exact test with FDR correction. Genes are rank-ordered according to significance of enrichment (Fisher’s exact test false discovery rate), and top 5 genes are indicated with gene symbols.

See also Figure S3 and Table S7.
Figure 5. LPA pathway inhibition attenuates fibrosis progression and reduces HCC in cirrhosis-driven HCC rat model

(A) Plasma autotaxin (ATX) activity over time during liver fibrosis progression in low-dose diethylnitrosamine (DEN) rat compared to control animals treated with PBS.

(B) Hepatic lysophosphatidic acid receptor 1 gene (Lpar1) expression (normalized to Actin) over time during liver fibrosis progression.

(C) Macroscopic images of the livers and tumors, H & E staining (arrow heads indicate tumor, scale bar indicates 100 µm), trichrome stain of fibrosis (scale bar indicates 250 µm), and α-smooth muscle actin (SMA) (marker of activated hepatic stellate cells, scale bar indicates 250 µm).

(D) Change in collagen proportionate area by AM063 or AM095 treatment.

(E) Change in histological liver fibrosis score, Ishak score (Ishak et al., 1995), by AM063 or AM095 treatment.

(F) Change in number of HCC nodules by AM063 or AM095 treatment.

permutations (1,000 times). GSEI of +3 indicates induction (orange) at enrichment p=0.001, GSEI of −3 indicates suppression (green) at enrichment p=0.001, and GSEI of 0 indicates no modulation (white) at enrichment p=1.0.

Each experiment was performed at least in three biological replicates, and the results are presented by mean and standard deviation (error bar). p values were calculated by t-test with Bonferroni correction.

See also Figure S4.
Figure 6. HCC risk gene signature modulation by LPA pathway inhibition by AM095 in organotypic ex vivo culture of clinical fibrotic liver tissues
HCC risk gene signature prediction was determined using tissues before the treatment. Modulation of HCC risk gene signature and hepatic stellate cell signature by AM095 is presented as gene set enrichment index (GSEI). Modulation of the signature member genes is presented as log2-fold change compared to respective DMSO-treated controls. Orange and green colors in the upper heatmap indicate induction and suppression of gene signature by GSEI, respectively. GSEI of +3 indicates induction (orange) at enrichment p=0.001, GSEI of −3 indicates suppression (green) at enrichment p=0.001, and GSEI of 0 indicates no
modulation (white) at enrichment p=1.0. Read and blue colors in the lower heatmap indicate up- and down-regulation of gene expression, respectively. See also Figure S5.