Experimental and Molecular Pathology 111 (2019) 104319

Contents lists available at ScienceDirect

Experimental and Molecular Pathology

journal homepage: www.elsevier.com/locate/yexmp

Identification of prognostic biomarker in predicting hepatocarcinogenesis from cirrhotic liver using protein and gene signatures

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ARTICLE INFO

Keywords:
Hepatocellular carcinoma
Liver cirrhosis
Caveolin-1
Reverse-phase protein array

ABSTRACT

Introduction: Cirrhosis primes the liver for hepatocellular carcinoma (HCC) development. However, biomarkers that predict HCC in cirrhosis patients are lacking. Thus, we aimed to identify a biomarker directly from protein analysis and relate it with transcriptomic data to validate in larger cohorts.
Material and method: Forty-six patients who underwent hepatectomy for HCC that arose from cirrhotic liver were enrolled. Reverse-phase protein array and microarray data of these patients were analyzed. Clinical validation was performed in two independent cohorts and functional validation using cell and tissue microarray (TMA).
Results: Systematic analysis performed after selecting 20 proteins from 201 proteins with AUROC > 70 effectively categorized patients into high ($n = 20$) or low ($n = 26$) risk HCC groups. Proteome-derived late recurrence (PDLR)-gene signature comprising 298 genes that significantly differed between high and low risk groups predicted HCC well in a cohort of 216 cirrhosis patients and also de novo HCC recurrence in a cohort of 259 patients who underwent hepatectomy. Among 20 proteins that were selected for analysis, caveolin-1 (CAV1) was the most dominant protein that categorized the patients into high and low risk groups ($P < .001$). In a multivariate analysis, compared with other clinical variables, the PDLR-gene signature remained as a significant predictor of HCC (HR 1.904, $P = .01$). In vitro experiments revealed that compared with mock-transduced immortalized liver cells, CAV1-transduced cells showed significantly increased proliferation ($P < .001$) and colony formation in soft agar ($P < .033$). TMA with immunohistochemistry showed that tissues with CAV1 expression were more likely to develop HCC than tissues without CAV1 expression ($P = .047$).
Conclusion: CAV1 expression predicts HCC development, making it a potential biomarker and target for preventive therapy.

1. Introduction

Hepatocellular carcinoma (HCC) accounts for about 75% of liver cancers (Ferlay et al., 2015). Despite recent advances in the diagnosis and management of HCC, HCC prevalence and mortality remain high owing to a lack of early markers of the disease. Chronic liver inflammation and persistent liver injury with repeated regeneration prime the organ for HCC development. Cirrhosis is a well-established risk factor for HCC, with > 80% of HCCs arising in the background of cirrhosis (Society, A. C, 2016) and 3–8% of patients with this cancer-prone microenvironment develop HCC each year (Bruix et al., 2011). Surveillance for early-stage HCC in at-risk patients involves noninvasive imaging and laboratory tests (Bruix et al., 2011). Although surveillance tests enable the early detection of HCC, they neither predict HCC development nor the prognosis of patients with cirrhosis. The molecular mechanisms of early hepatocarcinogenesis are largely unknown, and most studies have focused on elucidating these mechanisms in fully developed tumors only. Since liver microenvironment plays an important role in contributing HCC initiation and progression, identifying the genes that dysregulate liver microenvironment and that promote hepatocarcinogenesis is important.

We recently identified genomic predictors of de novo recurrence after surgical resection that are associated with hepatic injury and regeneration (Kim et al., 2014). In addition, Hoshida et al. (Hoshida et al.,

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https://doi.org/10.1016/j.yexmp.2019.104319
Received 15 July 2019; Received in revised form 16 September 2019; Accepted 13 October 2019

2013) found a genomic expression profile derived from surrounding non-tumor liver tissue that predicted HCC in patients with hepatitis C–related early-stage cirrhosis. These analyses were based on gene expression data. Although the measurement of gene sequences and expression levels is reliable and relatively simple, it does not directly reflect the activities of signaling proteins. Indeed, recent research has revealed that gene expression has only a partial concordance with protein expression and that gene expression alone does not accurately predict the state of protein signaling pathways driven by post-translational modifications (Vogel and Marcotte, 2012). Furthermore, compared with DNA, mRNA, miRNA, and other nucleic acid content, protein-level regulation and post-translational modification more profoundly affect cells.

Reverse-phase protein microarrays (RPPA) is a high-throughput method that can concurrently test hundreds of different proteins and pathways at different expression levels and posttranslational modifications, including cleavage and activated phosphorylated states (Iadevaia et al., 2010). For the past few years, RPPA had been an alternative to conventional immunoblotting. By coupling RPPA with mRNA expression data, we aimed to identify biomarker that could identify liver cirrhosis patients who are in need of closer surveillance.

2. Material and methods

2.1. Patient cohorts

As a training set, this retrospective study included 46 patients with HCC that occurred in cirrhotic liver background and had undergone hepatectomy between 2007 and 2011 (Table 1). Archived tumor tissues and matched surrounding non-tumor liver tissues were used for RPPA and gene expression analysis.

For validation, two cohorts were used to test the robustness of the proteomic derived predictor identified in the training cohort. Cohort 1 consisted 216 patients with histologically confirmed hepatitis C–related liver cirrhosis (GSE 15654) (Hoshida et al., 2013) while cohort 2 was comprised of 259 HCC patients who had undergone hepatectomy and surrounding non-tumor liver tissues were used to evaluate development of HCC.

2.2. Reverse phase protein array

The lysates were prepared to provide 1.5 μg/uL of total protein lysate and RPPA assay was performed at the Functional Proteomics RPPA Core Facility at MD Anderson, as previously described (Iadevaia et al., 2010). Briefly, serial diluted protein lysates were arrayed onto nitrocellulose-coated slides using an Aushon 2470 Arrayer (Aushon BioSystems, Billerica, MA, USA), including the spots corresponding to positive and negative controls prepared from mixed-cell lysates and dilution buffer, respectively. Each slide was probed with 201 validated primary antibodies followed by detection with appropriate biotinylated secondary antibodies. The slides were scanned, analyzed, and quantified using Array-Pro Analyzer software (MediaCybernetics) to generate spot intensity. SuperCurve GUI (Hu et al., 2007) was used to estimate relative protein levels (in $\log_2$ scale). The data was collected and normalized using software specifically developed for RPPA analyses (http://www.vigenetech.com). Replicate data were averaged, $\log_2$-median centered, hierarchically clustered (Cluster 3.0), and visualized in heatmaps (Java TreeView 1.1.1).

2.3. Gene expression data from human tissues

Gene expression data from the training cohort and cohort 2 were generated using Illumina microarray platform HumanHT-12. For cohort 1, whole-genome gene expression profiling was performed using the complementary DNA–mediated annealing, selection, extension, and ligation (DASL) assay (Illumina) (Hoshida et al., 2013). The expression level of each gene was transformed to $\log_2$ base and mean-centralized before further analysis.

2.4. Immunohistochemistry

TMA blocks of surrounding non-tumor liver tissues obtained from 217 HCC patients who had hepatectomy and were followed up for at least 3 years were used for immunohistochemistry (IHC) validation. Primary antibodies against CAV1 (CST and D46G3; 1:250 dilution) were used. For interpretation of the IHC staining results, staining intensity was graded on a scale of 0 to 3 (0, negative; 1, low; 2, intermediate; and 3, high) and distribution extent was rated on a scale of 0 to 3 (0, < 1% positive, 1, 1–9% positive; 2, 10–19% positive; 3, > 20% positive). Positive CAV1 staining was defined as an intensity score of 3 and a distribution score of at least 2 (Supplementary Fig. 1).

2.5. Analysis of training cohort proteomics and genomics data

Intrahepatic recurrence after hepatectomy for HCC is defined as de novo tumor or metastasis depending on whether it occurs < 2 or ≥ 2 years after the treatment (Imamura et al., 2003). In our study, we set the cutoff time at which recurrence would be considered to be de novo HCC as 3 years after hepatectomy in order to define newly developed HCC with greater confidence. The area under the receiver operating characteristic curve (AUROC) of 201 proteins predictive of HCC were evaluated. Twenty protein features with AUROC > 0.7 and $P < .05$ were selected. The expression data of these 20 protein features were used for unsupervised clustering to divide patients into high or low risk of developing HCC.

To identify mRNAs whose expression is significantly associated with either two proteomic subtypes, mRNA expression data from the same tissues were generated. The gene expression data were analyzed using the BRB ArrayTools software program (Simon et al., 2007). Genes whose expression was significantly different between the 2 subtypes were selected were identified using *t*-test ($P < .01$ and $\log_2$-based ratio > 0.1). This gene signature was used for prognostic validation of the 2 subtypes in independent cohorts by applying Bayesian compound covariate predictor (BCCP) model (Fig. 1) (Radmacher et al., 2002).

The robustness of the newly developed gene signature was compared with two previously reported gene signatures; 186-gene Broad signature and the hepatic injury and regeneration (HIR) signature using Cox regression model.

2.6. Network analysis

For pathway analysis, the gene signature was uploaded into the Ingenuity Pathways Analysis software (Ingenuity Systems, Redwood City, CA, USA; http://www.ingenuity.com/) and the enriched gene sets and upstream regulators in each subtype was analyzed.

2.7. In vitro analysis

2.7.1. Cell culture and reagents

Human immortalized hepatocyte LO2 cells were cultured in 10% fetal bovine serum/RPMI 1640 with 25 mM HEPES supplemented with 100 U/mL penicillin and 100 mg/mL streptomycin. The rabbit monoclonal CAV1 antibody (D46G3) and rabbit monoclonal c-Myc antibody (D84C12) were purchased from Cell signaling (Danvers, MA, USA). The β-actin (sc-47,778) was purchased from Santa Cruz Biotechnology (Dallas, TX, USA).

2.7.2. Plasmid constructs and stable cell lines

The CAV1 cDNA fragment containing the BamH1F and Xho1R restriction site was amplified by polymerase chain reaction from the human HCC cell line SNU 449 (forward primer: 5′-TCTCTACACCGTTCCCATC-3′; reverse primer: 5′-CAAATGCCGTCAAAACTGTG-3′). The

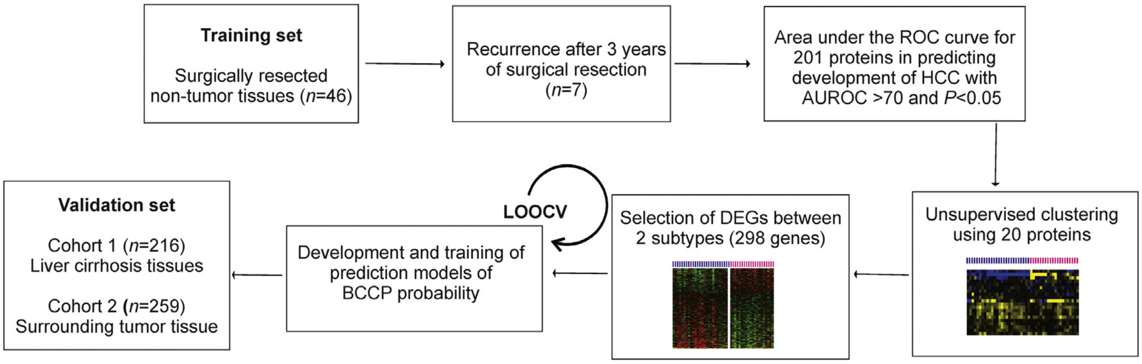

Fig. 1. Schematic overview of the construction of Bayesian compound covariate predictor models.

amplified fragment was cloned into a pENTER commercial vector (Life Technology) and then subcloned into a pCDHblast MCSNard lentiviral plasmid.

After the CAV1 sequence was verified, the lentivirus was transfected with PMD2G and PsPAX2 plus pCDHblast MCSNard (mock) or 8 pCDHblast/Cav1 (pCAV1) into 293 T cells. The medium was changed after 24 h, and supernatant harvested 48 h after transfection was used to transduce LO2 cells.

2.7.3. *In vitro functional assays*

The in vitro tumorigenic ability of CAV1 was assessed with an XTT cell proliferation assay, a foci formation assay, and a colony formation in soft agar assay. For the foci formation assay, 1000 cells were seeded into 6-well plates. For the colony formation in soft agar assay, 5000 cells suspended in 0.3% agar were gently added on top of bottom agar layer that was prepared with 0.6% low-melt agarose. After 21 days of culture, colonies were stained with 0.05% crystal violet and were examined microscopically. All experiments were done in triplicate.

3. Results

3.1. *Proteins associated with late HCC recurrence*

Proteins associated with de novo HCC (i.e., recurrence occurring 3 years after resection) were selected by applying an AUROC approach to proteomic data from the surrounding non-tumor liver tissues of HCC patients. Of the 46 HCC patients in the training cohort, 7 developed HCC 3 years after hepatectomy and 20 proteins were significantly associated with late recurrence (Supplementary table 1). As expected, clustering of the data of these 20 proteins dichotomized patients into 2 subgroups at low or high risk of HCC. Of the 7 patients who had late recurrence, 6 (86%) were grouped into a small cluster (Fig. 2A). Interestingly, the most prominent protein in the high-risk group was caveolin-1 (CAV1).

3.2. *Identification and validation of a gene signature of late HCC recurrence*

Proteome-derived late recurrence (PDLR) gene signature were derived using unique genes (298 genes) with expression that differed significantly between the high- and low-risk HCC groups ($P < .01$ and $\log_2$ ratio > 0.1) (Fig. 2B). Among these 298 genes, *FOSB*, *JUN*, *KLF6*, *CTNNB1*, and *WEE1* were upregulated in the high-risk group.

We next determined whether the PDLR gene signature could predict HCC in validation cohorts. Cohort 1 shared 218 genes with the training cohort. Application of the BCCP algorithm to these expression data yielded a PDLR gene signature that robustly stratified 89 patients (41.2%) into a high-HCC risk group and 127 patients (58.8%) into low risk group (Fig. 3A). Kaplan-Meier analysis showed that compared with the low-risk PDLR group, the high-risk PDLR group had a significantly higher HCC risk 3 years after hepatectomy ($P = .021$ by log rank test). Consolidation of validation was done in cohort 2. When 180 genes shared between cohort 1 and training cohort were analyzed, 89 patients (34.4%) were predicted to have a high probability (> 0.5) of grouping to high risk group and this high risk group of patient had a significantly higher risk of developing HCC 3 years after hepatectomy than the low risk group ($P = .022$). (Fig. 3B).

3.3. *Clinical significance of the PDLR gene signature*

The PDLR gene signature remained clinically significant even when it was analyzed with other clinical factors related to hepatic deterioration (HR 1.904, $P = .01$) and with previously validated prognostic models, including the 186-gene Broad signature and the HIR gene signature (HR 1.997, $P = .004$) (Table 2).

3.4. *Biological significance of the PDLR gene signature*

We next investigated the effect of ectopic CAV1 on LO2 cells (human immortalized hepatocytes). Compared with mock-transduced LO2 cells, CAV1- transduced LO2 cells had significantly increased proliferation ($P < .001$; Fig. 4A), foci formation ($P = .026$; Fig. 4B), and colony formation ($P = .013$; Fig. 4C). These findings suggest that CAV1 stimulates hepatocyte proliferation.

To determine the role of CAV1 in human hepatocarcinogenesis, we evaluated CAV1 expression in surrounding non-tumor tissue from TMA of 217 patients who were followed up for > 3 years. Of these 217 patients, 63 (29%) had tissues with positive CAV1 staining, and 154 (71%) had tissues with negative CAV1 expression according to classification mentioned in the method. The rate of HCC development at 3 years after surgical resection among patients with CAV1 expression (24% [15/63]) was significantly higher than that of patients with no CAV1 expression, (12% [19/154], $P = .035$). Similarly, cumulative incidence of HCC development was significantly higher in CAV1 positive group than negative group ($P = .042$; Supplementary Fig. 2).

Next, the correlation between CAV1 expression levels and genes that are commonly mutated in human HCC were further analyzed. Significant positive correlation was observed between CAV1 expression with MYC (Pearson's $r = 0.306$, $P < .001$), TP53 (Pearson's $r = 0.381$, $P < .001$), CTNNB1 (Pearson's $r = 0.367$, $P < .001$) and CCND1 (Pearson's $r = 0.157$, $P = .018$) expression while negative correlation was observed with AXIN1 (Pearson's $r = -0.204$, $P = .002$) expression.

Molecular analyses was used to gain insight into the biological significance of the PDLR gene signature. Categorizing the genes in the PDLR signature based on information of Ingenuity Knowledge Base repository revealed that the PI3K/Akt, Agrin interactaction at neuromuscular junction, HGF, and TREM1 canonical pathways were significantly activated (z-score > 2; Supplementary Fig. 3). As expected, most of the activated genes were related to cellular proliferation, cell death, and the cell cycle (Supplementary table 2). This suggests that the PDLR gene signature, which is mainly derived from CAV1 expression,

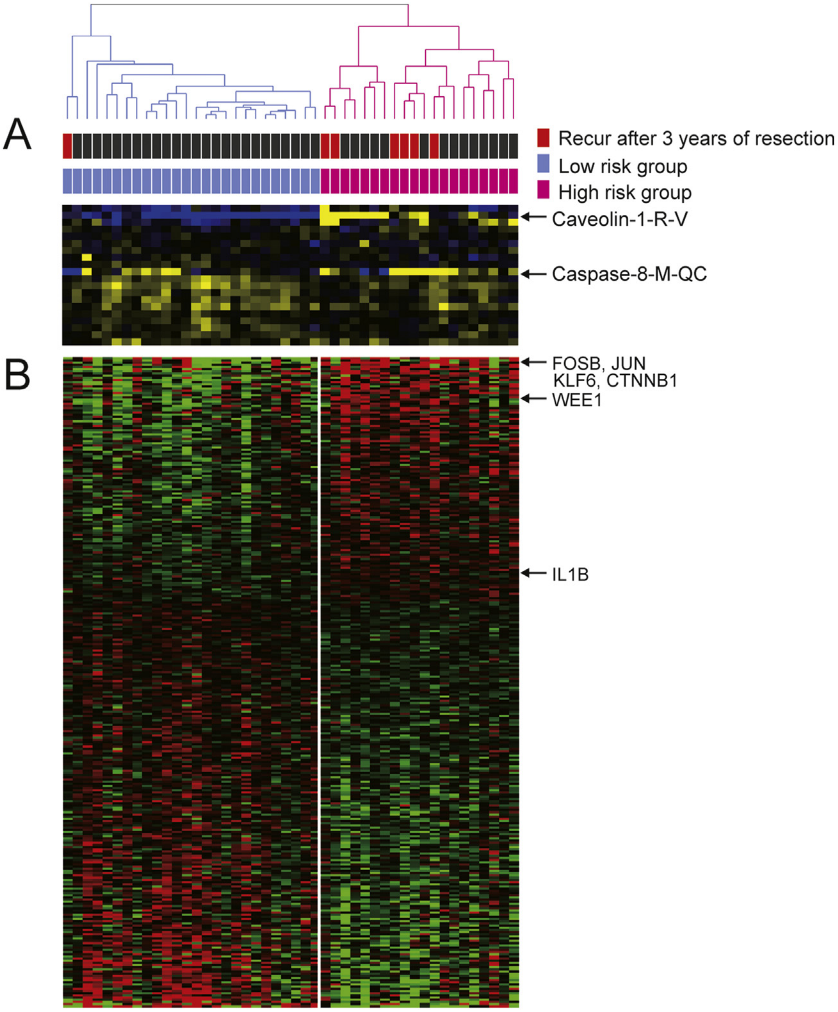

Fig. 2. Proteomics signature and PDLR gene signature.
(A) Unsupervised clustering of 20 protein features significantly associated with de novo HCC (HCC recurrence after 3 years of resection (B) Supervised clustering of 298 genes whose expression differed significantly between the low- and high-risk PDLR groups.

reflects increased cell proliferation, cell survival, and cell cycle capabilities, which promote hepatocarcinogenesis. Notable upstream regulatory genes that were significantly activated (z-score ≥ 2) were TNF, VEGF, ERK1/2, IL6, TGFB-1, EGFR, and FGF1/2 (Supplementary table 3). These gene are involved in the pathogenesis of liver fibrosis and the regulation of inflammation, which could have driven hepatocarcinogenesis.

4. Discussion

Our findings show that CAV1 was distinguishably overexpressed in a group at high risk for HCC. Although ours is not the first study to identify a biomarker of cirrhotic liver that will progress to HCC, it is the first to investigate protein expression levels in cirrhotic liver.

RNA is a primordial molecule, but the measurement of protein changes is more useful for elucidating human disease processes (Wilhelm et al., 2014). Before the advances in next-generation DNA sequencing and proteomics, it was assumed that transcript abundances are the main determinant of protein. However, mRNA and proteins often showed squared Pearson correlation coefficient of ~0.4 only, reflecting that only 40% of the variation in protein concentration is explained by mRNA abundance (Maier et al., 2009). Post transcriptional processes are key to the final synthesis of protein and protein degradation contribute to variation in protein concentration rather than transcription and degradation of mRNA (Plotkin, 2010). On the basis of these findings, which suggest that protein level and expression data are important in determining disease progression, we identified CAV1 as a protein whose expression is associated with HCC development. Our analysis revealed that patients could be neatly categorized as having either a high or low risk of HCC mainly on the basis of CAV1 expression.

CAV1, a structural protein of caveolae in the plasma membrane of many specialized cells, including endothelial cells, fibroblasts, epithelial cells, and adipocytes, has been known to regulate liver function. The expression of CAV1 is different on different tumors; it is downregulated in ovarian, lung and mammary carcinomas while it is upregulated in bladder, esophagus and prostate carcinoma (Fernandez-Rojo and Ramm, 2016). CAV1 expression is elevated in cirrhotic liver but is hardly detectable in normal liver (Yokomori et al., 2003), and several studies have shown that it plays a role in HCC progression and metastasis (Mao et al., 2016; Moreno-Caceres et al., 2014; Yu et al., 2014). Increased CAV1 expression is reported to increase the proliferation ratio, metastatic potential, and migration capacity of hepatoma cell lines (Cokakli et al., 2009). CAV1 also has a role in HCC progression and metastasis, which involve the Wnt/β-catenin pathway (Yu et al., 2014) and FAK/Src signaling pathway (Meyer et al., 2013). β-catenin is a subunit of the cadherin protein complex on the cellular surface which acts as a signaling molecule in the Wnt pathway. AXIN acts as the scaffold of this complex and when AXIN relocates to the plasma membrane, β-catenin is stabilized and freely translocate to the nucleus increasing transcription of genes involved in cell growth (Nusse and

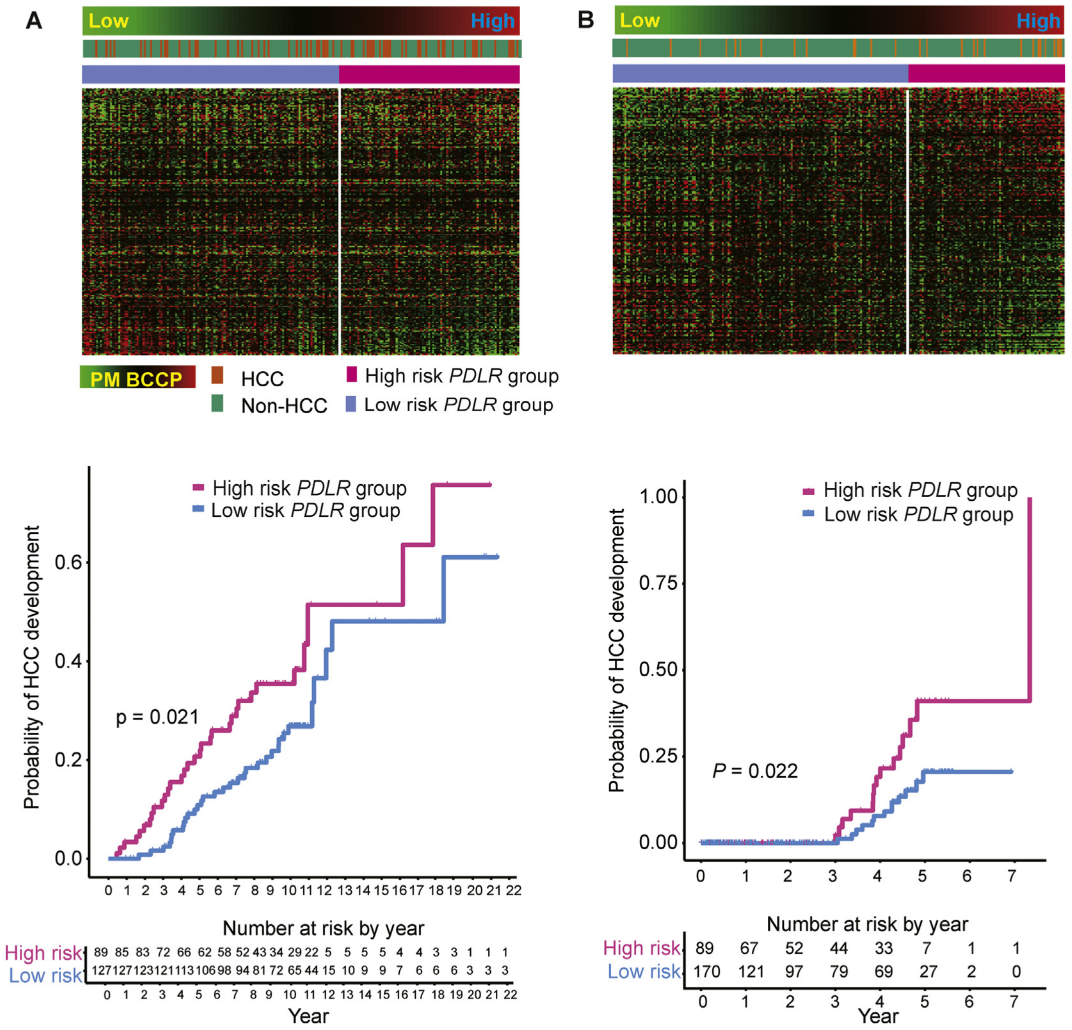

Fig. 3. Heatmap and Kaplan-Meier plot for HCC development in cohorts 1 and 2.
Heatmap illustrating the supervised clustering of (A) 218 genes shared between the training cohort and cohort 1 and (B) 180 genes shared between the training cohort and cohort 2. The high risk PDLR gene signature was associated with lower survival rate in both cohorts.

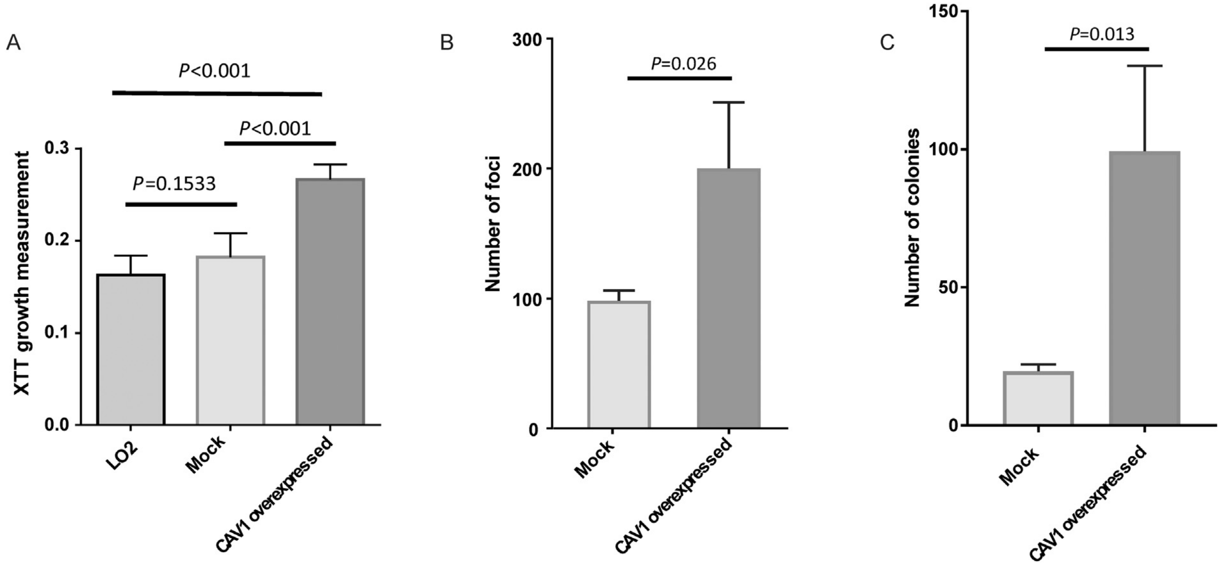

Fig. 4. In vitro tumorigenic abilities of CAV1 in LO2 cells.
(A) XTT cell proliferation assay, (B) foci formation assay, and (C) soft agar colony formation assay show that CAV1-transduced cells were significantly more tumorigenic than mock-transfected cells.

Clevers, 2017). Our present study showed positive correlation between CAV1 and CTNNB1 gene expression while negative correlation between CAV1 and AXIN1 expression which supports previous studies. In addition, other studies have shown that exosomes release CAV1 in rats with liver disease but not in healthy rats (Conde-Vancells et al., 2008), as well as in patients with alcoholic liver disease (Gao et al., 2014), suggesting that CAV1 is released under pathological conditions.

Although many studies have investigated the role of CAV1 in HCC aggressiveness, its roles in the transformation of hepatocytes to cancer cells in the cirrhotic liver remain largely unknown. The present study's findings indicate that CAV1 overexpression indeed initiates hepatocarcinogenesis along with genes that are frequently mutated in human HCC such as MYC, TP53, CTNNB1, CCND1 and AXIN1. An association between CAV1 and MYC in hepatocarcinogenesis has not been reported, but their association in prostate cancer has been studied (Yang et al., 2012). One previous study suggested that CAV1 plays a role in Akt activation, which inhibits the MYC-induced apoptosis that helps mouse prostatic intraepithelial neoplasia transition to prostate cancer (Li et al., 2003). Although further investigation is required to determine the extent to which this mechanism can be applied to hepatocarcinogenesis, our gene network analysis showed that one of the significantly activated pathways was the PI3K/AKT pathway, which suggests that CAV1 overexpression can activate AKT and to increase cell proliferation. Another interesting finding was the activation of Agrin interaction at neuromuscular junction which is not well studied in HCC. Agrin, utilized by motor neurons to stimulate the LRP4-MuSK receptor in muscles for neuromuscular junction (NMJ) formation is not confined to NMJ only but rather is expressed in other tissues and organs such as brain, kidney and bones. Despite the unknown function of Agrin in HCC, proteomic study by Hong et al. discovered its overexpression in HCCs and reported the role of Agrin in cellular migration, invasion and tumor growth through activation of Yes-Associated Protein (YAP) (Chakraborty et al., 2015; Chakraborty et al., 2017). Activation of YAP by suppressing Hippo pathway and stimulation of integrin-focal adhesion, promotes liver cancer development. In addition, since CAV1 is the structural protein component of caveolae of the plasma protein, it may induce agrin and promotes hepatocarcinogenesis.

The limitation of this study is that the proteomic signature was not directly validated in test cohorts owing to a lack of RPPA data for the validation cohort. Therefore, the PDLR gene signature was derived from genes with significantly different expression between the groups at high or low risk for HCC and the robustness of the PDLR gene signature was validated in 2 cohorts with sufficient numbers of patients. The prognostic ability of the PDLR gene signature was also compared with those of previously validated gene signatures, including 1) the 186-gene Broad signature, which is derived from the surrounding non-tumor liver tissues of HCC patients and is significantly associated with late HCC recurrence (Hoshida et al., 2008), and 2) a 233-gene expression signature derived from human liver undergoing hepatic injury and regeneration (the HIR signature) (Kim et al., 2014). The PDLR gene signature shared only 1 gene (CCT8) with the Broad signature and 6 (MCL1, ARHGEF5, C13orf15, WEE1, PLAUR, and JUN) with the HIR gene signature. This reflects that the PDLR gene signature is a unique gene signature distinguishable from those previously reported.

In summary, we have shown without bias that CAV1 plays an important role in initiating hepatocarcinogenesis and thus could be used as a biomarker for identifying cirrhosis patients at high risk for HCC. Furthermore, the validation of CAV1 as disease biomarker could be expanded to include liquid biopsy, which is repeatable and less invasive than liver biopsy to detect HCC at an early stage.

Funding

This study was supported by Duncan Cancer Prevention Research Seed Funding Program at The University of Texas MD Anderson Cancer Center (2016 cycle) and the National Institutes of Health through MD Anderson's Cancer Center Support Grant P30 CA016672. Additional support was provided by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education (NRF-2018R1D1A1B07040523) and Korea University Anam Hospital (Grant No. O1800751).

Declaration of Competing Interest

The authors declare no conflict of interests.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.yexmp.2019.104319.

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