

===== BIOINFORMATICS =====

Integrative Bioinformatics Analysis of Liver Transcriptomics and Serum Fibroblast Growth Factor 21 Protein Levels in Monitoring Progressive Fibrotic Liver Disease

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Abstract. Advanced fibrosis defines mortality risk in chronic liver disease. Current diagnostics fail to capture the molecular transition from early injury to permanent scarring. They often rely on binary classifications obscuring the biological drivers of disease progression. We mapped the gene expression profiles differentiating advanced (F3-F4) from early (F0-F2) fibrosis to determine their cellular origin and protein-level translatability across distinct disease etiologies. We analyzed public bulk RNA-sequencing data (E-MTAB-6863) to isolate genes distinguishing early from advanced fibrosis. We then deconvoluted these signals using single-nucleus RNA-sequencing (snRNA-seq; GSE179548) to identify specific cell types. We validated candidates proteomically in a multi-omics hepatocellular carcinoma dataset comparing viral versus metabolic etiologies. We identified a 105-gene signature associated with advanced fibrosis enriched for extracellular matrix organization and epithelial development. snRNA-seq localized this signal primarily to cholangiocytes rather than hepatic stellate cells indicating a ductular reaction. Proteomic validation showed a divergence. Structural markers (COL1A1, THY1) remained elevated regardless of etiology. Metabolic and ductular markers (AKR1B10, KRT23, SPP1) were specific to metabolic conditions (NAFLD alcoholic liver disease). Advanced fibrosis is fueled by a biliary epithelial response not just matrix deposition. The fibrotic scaffold is conserved across patients. The accompanying metabolic adaptations are etiology dependent. This suggests that effective biomarkers must be tailored to the specific metabolic context of the disease rather than relying on a universal fibrosis panel.

Key words: *liver fibrosis, cholangiocytes, ductular reaction, multi-omics, etiology-specific biomarkers*

INTRODUCTION

Chronic liver disease spans metabolic dysfunction-associated steatotic liver disease to viral hepatitis. It remains a persistent global health burden [1–3]. The convergent outcome of these injuries is hepatic fibrosis. Fibrosis is a reparative response defined by excessive extracellular matrix (ECM) deposition and architectural collapse [4]. Fibrosis severity dictates the risk of decompensated cirrhosis and hepatocellular carcinoma. Accurate staging is the primary determinant of patient prognosis [5].

There were very few targeting to staging of fibrosis sensitively with clinical methods available so far, which could not satisfy the demand of individual therapeutic. Percutaneous liver biopsy is the traditional reference standard but it is problematic because of its invasiveness,

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sampling error and inter-observer variability. *Ex-vivo* noninvasive tests (NITs) decrease the requirement of biopsy. They are based on binary categorizations distinguishing minimal fibrosis (F0-F1) from advanced disease (F3-F4) [6]. These strategies result in a large diagnostic deficit. This void impedes the identification of the molecular changes that lead to progression from early injury to permanent scarring. Serological markers of fibrosis are commonly indirect consequences of liver damage rather than the specific underlying molecular processes governing fibrogenesis [7, 8].

There is a transition from measuring level of a morphological characteristic to measuring molecular level through high-throughput transcriptomics and proteomics [9, 10] but there are still many areas of knowledge lacking. Specific gene expression changes in end-stage cirrhosis can be determined but profiling the different stages between advanced fibrosis and early stages of disease needs further analysis. There are no tissue types that support analyses for finding connections between tissue-level gene expression and more downstream circulating protein levels; their place of production is hidden in larger tissue data sets of volume [11]. To identify reliable, non-invasive biomarkers for high fibrogenic pathway expression in tissue will require a direct correlation to detection of a protein fingerprint (biomarker protein) to a good source of the biomarker protein. Knowing what cell type produces these biomarker proteins is important.

The necessity to treat this complicated fibrotic biology on multiple scales with a more precise and detailed manner results from its very complex nature [12]. Fibrosis is the result of the convergence of 3 parties: an injured hepatocyte, an activated HSC, and an immune cell that has undergone the process of migration into the injured area [13, 14]. The average analysis across a large volume of tissue will obscure the distinct signatures. This could obscure signals from strong effectors. Biological regulation is non-linear. Post transcriptional processes frequently uncouple mRNA abundance from effective protein output [15, 16]. A gene signature is not enough. It is a biological fact that these transcriptional programs are translated into durable, detectable proteins.

This paper uses a multi-dimensional analytic model to examine these gaps in knowledge. The main goal is to delineate the DE gene profile that exclusively discriminates advanced fibrosis from early-stage diseases. This study identifies highly up-regulated fibrogenic pathways. It then assesses them at the single-cell level to identify exactly which cells are contributing. The present study aims at determining whether these transcriptomic signals are also reflected at proteomic levels and if they remain consistent across a wide variety of etiologies. This supports that they may be used as general biomarkers to monitor advancing liver disease.

METHODOLOGY

Advanced vs. Early Liver Fibrosis Differential Gene Expression Analysis

To characterize transcriptional changes in progressive fibrosis, a RNA-sequencing cohort was retrieved from the ArrayExpress database (www.ebi.ac.uk/biostudies/arrayexpress) with accession ID E-MTAB-6863. This cohort consists of a dataset from liver biopsies from 69 patients with chronic liver disease, including Hepatitis C Virus (HCV) and Fatty Liver Disease (FLD). We stratified patients by histological fibrosis stage (METAVIR), categorizing samples into Early Fibrosis (F0-F2) and Advanced Fibrosis (F3-F4). Then, differential expression analysis was performed using the DESeq2 R package [17] between the Advanced Fibrosis group and the Early Fibrosis group to isolate progression-specific signals. However, statistical threshold of an adjusted p -value (p_{adj}) < 0.05 and biological threshold of absolute $\log_2$ fold change > 1 was used to obtain the upregulated genes shortlisted which were used for further analysis.

Functional Enrichment Analysis

We determined biological pathway associations by querying the EnrichR database [18]. We analyzed the upregulated signature against three primary ontologies: Gene Ontology (GO) for biological processes, Kyoto Encyclopedia of Genes and Genomes (KEGG) for signaling pathways, and Reactome for molecular reaction networks. We considered enrichment significant at a $p_{\text{adj}} < 0.05$. Priority was given to the terms involving extracellular matrix organization, cell migration, and epithelial development for validation.

Single-Nucleus RNA-seq Validation

To identify the cellular sources of the fibrotic signature, we analyzed independent single-nucleus RNA-seq (snRNA-seq) data (GSE179548) from the Gene Expression Omnibus (GEO). This dataset consists of 5357 nuclei isolated from whole-liver cirrhotic tissue obtained during autopsies of four individuals (three males, one female; aged 70–83 years). Libraries were sequenced on an Illumina NovaSeq 6000 platform (S4 flow cell). We analyzed raw data with the Seurat R package. We filtered out doublets and low-quality nuclei using strict quality control filters and excluded nuclei with $> 5\%$ mitochondrial transcripts (indicative of ambient RNA/dying cells) or < 200 detected genes (empty droplets). We then conducted a UMAP dimensionality reduction and clustered cells at 0.9 resolution. We labeled different cell populations with an inbuilt automated classification algorithm, ScType [19]. To map the bulk derived signature onto the single cell data, we computed a composite Fibrosis Module Score with the AddModuleScore function of Seurat [20]. This function estimates the collective expression of upregulated gene set in each cell versus a control random-gene set, so that we can visualize pathway enrichment among different cell types using violin and box plots.

Proteomic Validation Across Etiologies

To validate the translation of gene expression findings to the protein level and assess their stability across different liver disease etiologies we analyzed a multi-omics hepatocellular carcinoma dataset [21]. We extracted protein expression levels (parts per billion, ppb) for a targeted panel of candidates. We categorized markers into structural/myofibroblastic (COL1A1, THY1) and ductular/metabolic (AKR1B10, KRT23, EPCAM, SPP1, ITGB6). We stratified expression data by disease etiology specifically comparing Alcoholic Liver Disease, Hepatitis B, Hepatitis C and NAFLD to determine if markers represented universal fibrotic features or etiology specific metabolic adaptations.

RESULTS

Advanced Fibrosis is Characterized by a Distinct Pro-Fibrotic Gene Signature

To identify the molecular signature of fibrosis progression, we performed DE analysis on hepatic RNA-seq data from 69 patients, comparing advanced-stage (METAVIR F3-F4) against early-stage (METAVIR F0-F2) liver fibrosis. This analysis identified a set of 105 significantly upregulated genes (adjusted p -value < 0.05) in the advanced fibrosis group (Fig. 1A). The most highly upregulated genes included MYH4 ($\log_2\text{FoldChange} = 4.21$, $p_{\text{adj}} = 0.0003$) and KRT23 (4.08, 0). Other strongly upregulated genes included EPCAM (2.23, 0), MMP7 (1.95, 0), SPP1 (1.42, 0.0001), and SOX9 (1.46, 0).

In line with the primary goal of the study, this upregulated gene set was highly enriched for canonical, pro-fibrotic genes, confirming a strong association with fibroblast activation and progressive matrix deposition. Key genes involved in ECM production and remodeling were significantly elevated, including COL1A1 (1.05, 0.0009), COL4A3 (1.18, 0.0001), COL15A1 (1.23, 0.0003), LOXL4 (1.60, 0), and VCAN (1.08, 0). We also observed significant upregulation of key signaling molecules and matricellular proteins, such as TGFB2 (1.17, 0), PDGFD (1.15, 0), LTBP2 (1.34, 0), and THY1 (1.16, 0.0001). This signature suggests a highly active and dynamic process of matrix deposition and tissue remodeling in advanced disease.

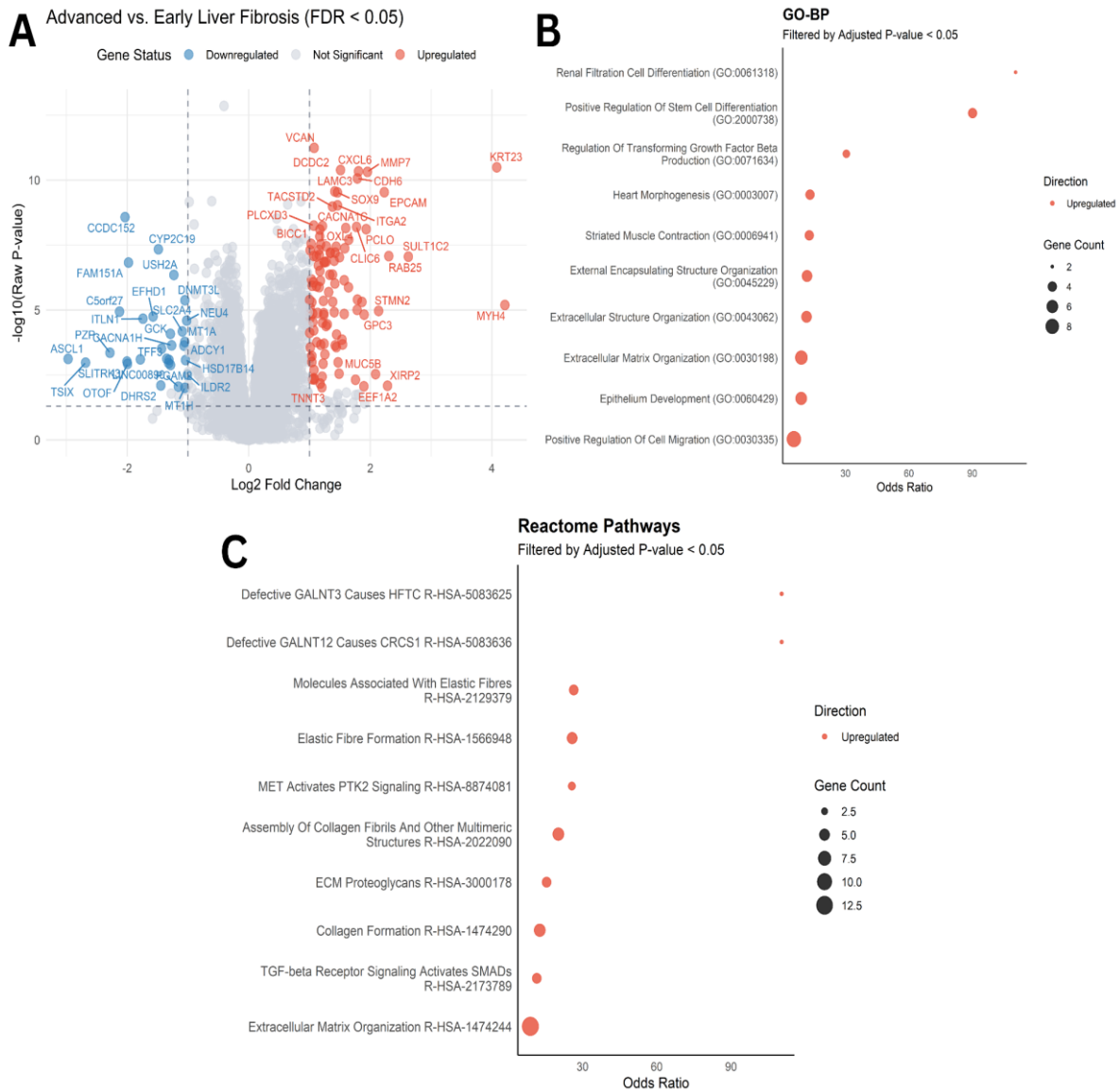


Fig. 1. A – Volcano plot displaying gene status (downregulated, not significant, upregulated) with $\log_2$ fold change against $-\log_{10}$ (raw p -value). **B** – Gene Ontology (GO) Biological Processes enrichment filtered by adjusted p -value < 0.05. **C** – Reactome Pathways enrichment filtered by adjusted p -value < 0.05

Pathway Enrichment Analysis Reveals an Active Pro-Fibrotic Network

To know the functional process that was affected by these deregulated expressions, Gene set enrichment analyses were conducted with GO, KEGG and Reactome database system. The findings indicated an overriding pattern of active ECM-associated signaling and cellular activity. The top enriched biological theme was Extracellular Matrix Organization in all three enrichment analyses (GO:0030198, 0.012; Reactome R-HSA-1474244, 4.73e-6). This activity was further determined by Reactome database which showed highest ranking terms were Assembly Of Collagen Fibrils And Other Multimeric Structures (2.10e-4) followed by Collagen Formation (1.18e-3) (Figures 1B and 1C).

These pathways were driven by the upregulation of key fibrosis genes identified in the DE analysis including COL1A1, COL15A1, COL4A3, LOXL4, MMP7, SPINT1 and VCAN. This indicates that advanced fibrosis may be characterized by a persistent and active program of ECM production and assembly. The analysis strongly suggested that the signaling pathways may potentially drive this matrix deposition. The KEGG pathway ECM-receptor interaction

(7.62×10^{-7}) was the most significant finding involving upregulated genes such as COL1A1, COL4A3, LAMC2, LAMC3, SPP1 and the integrins ITGA2, ITGB6 and ITGB8 (Fig. 2A).

This finding was strongly supported by GO terms like Integrin mediated Signaling Pathway (GO:0007229, 0.036) and KEGG's Focal adhesion (1.67×10^{-4}). This suggests a feed forward loop where the stiff fibrotic matrix is sensed by cells via integrins and focal adhesions, which in turn perpetuates pro-fibrotic signaling.

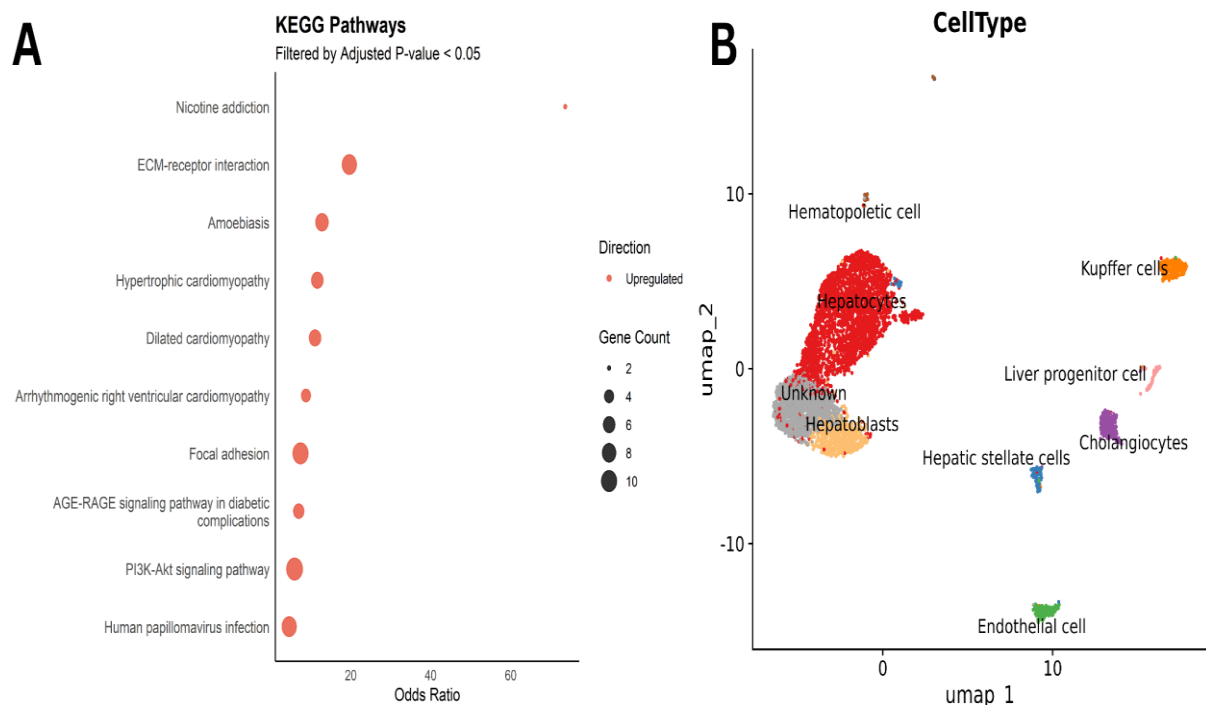


Fig. 2. A – KEGG Pathways enrichment analysis filtered by adjusted p -value < 0.05, showing upregulated pathways such as ECM-receptor interaction and Focal adhesion. **B** – UMAP dimensionality reduction visualizing distinct liver cell populations, including Hepatocytes, Cholangiocytes, Hepatic stellate cells, Kupffer cells, and others

The master regulator of fibrosis the TGF- β pathway was also highlighted. The GO term Regulation Of Transforming Growth Factor Beta Production (GO:0071634, 0.034) which includes the upregulated genes TGFB2, ITGB6 and ITGB8 was significantly enriched. This points to an active self-sustaining loop of TGF- β signaling in advanced disease. The downstream result of this activated signaling was evident in enriched terms related to cell behavior. We observed significant enrichment for Positive Regulation Of Cell Migration (GO:0030335, 0.020) with contributing genes including COL1A1, MMP7, PDGFD, TGFB2, SOX9 and LAMC2. This migratory phenotype is likely supported by the activation of intracellular growth and survival pathways as evidenced by the enrichment of the PI3K-Akt signaling pathway (KEGG, 3.13×10^{-4}). This pathway, which integrates signals from many of the upregulated genes (COL1A1, PDGFD, IGF2, SPP1 and integrins), is crucial for the proliferation and survival of activated HSCs and myofibroblasts.

Finally the enrichment of Epithelium Development (GO:0060429, 0.020) and Positive Regulation Of Stem Cell Differentiation (GO:2000738, 7.53×10^{-4}) driven by genes like SOX9, KRT23 and TACSTD2 (also known as EPCAM) suggests a potential role for a hepatic progenitor cell (ductular) reaction. This is a common histological feature in advanced liver disease and is associated with injury severity and a poor regenerative response.

SnRNA-seq Validation Pinpoints Cholangiocytes as the Source of the Fibrotic Signature

To de-convolute the bulk-tissue signature and identify the specific cellular drivers of the identified pathways, we did prioritize of shortlist genes with different fibrotic mechanisms. For

myofibroblast (HSC) activation, we chose to validate the canonical markers COL1A1, THY1 and LOXL4 and focused on KRT23, EPCAM and SOX9 for the strong epithelium development signal by hypothesizing a role of a ductular reaction. Furthermore, SPP1, ITGB6 and AKR1B10 were investigated to interrogate signaling/transcriptional signatures in specific metabolic/perturbation nodes connecting fibrosis with putative bile acid dysregulation.

We subsequently projected the expression profile of these genes as a module score onto an independent human liver fibrosis snRNA-seq dataset (GSE179548) from 5,357 cells of cirrhotic human livers across major liver cell types: hepatocytes, cholangiocytes, HSCs, Kupffer cells, endothelial cells, hepatic progenitor-like cells and hematopoietic/lymphoid (Fig. 2B). Evaluation of the fibrosis module score across these cell sorted populations indicated that the signature was overwhelmingly and nearly exclusively enriched in the Cholangiocyte population. Any other type of cell, such as Hepatocytes or HSCs, exhibited slight to no enrichment for this composite signature (Fig. 3A).

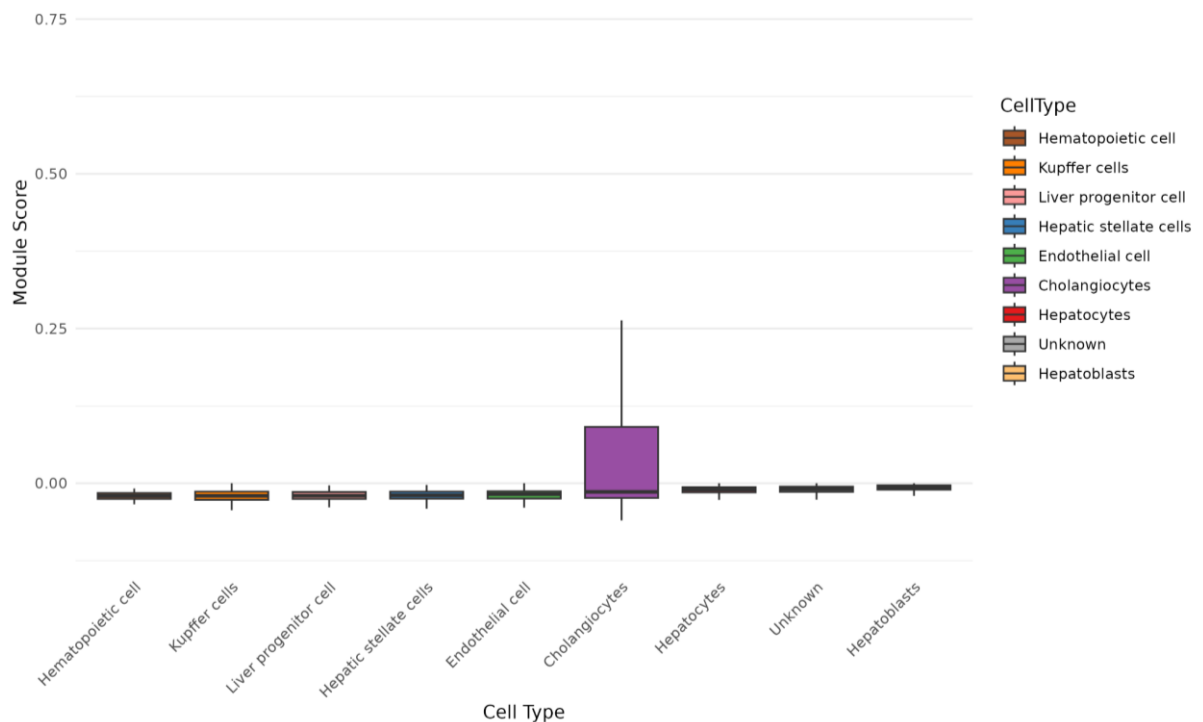


Fig. 3. Fibrosis Signature Score by Cell Type, displaying the distribution of module scores across different cell populations (Hematopoietic cells, Kupffer cells, Liver progenitor cells, Hepatic stellate cells, Endothelial cells, Cholangiocytes, Hepatocytes, Hepatoblasts), indicating specific enrichment in Cholangiocytes

This snRNA-seq analysis verifies our bulk pathway findings showing the Epithelium Development signature to be a major transcriptional driver in advanced fibrosis. The findings point to a pronounced cholangiocyte-derived ductular reaction as a major molecular change, rather than the canonical matrix production by HSCs.

Validation of Pro-Fibrotic Protein Expression across Diverse Liver Disease Etiologies

To determine if our shortlisted gene signature translates to the protein level and persists across distinct causes of chronic liver disease, we analyzed proteomic data [21] from an independent multi-omics cohort. We examined the protein expression levels (in parts per billion, ppb) of our key markers AKR1B10, COL1A1, THY1, ITGB6, KRT23, EPCAM, and SPP1 across various etiologies including alcoholic liver disease, hepatitis B/C, and NAFLD (Fig. 4).

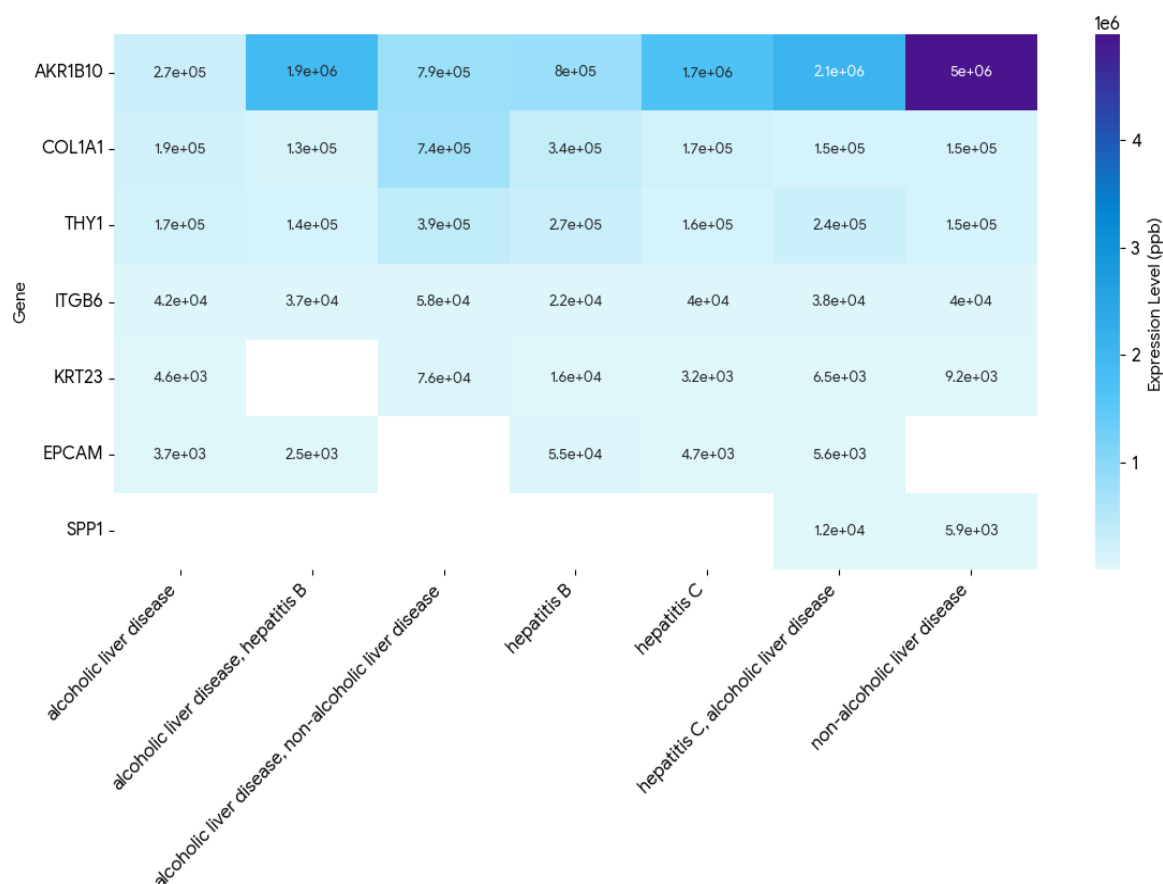


Fig. 4. Heatmap of protein expression levels (ppb) for specific genes (AKR1B10, COL1A1, THY1, ITGB6, KRT23, EPCAM, SPP1) stratified by disease etiology, including alcoholic liver disease, hepatitis B, hepatitis C, and non-alcoholic liver disease

There were two major patterns of expression identified. The first pattern is a consistent protein expression of the known fibrotic markers COL1A1 and THY1 within each of the disease backgrounds studied. This indicates that the core phenotype of matrix production is a common feature of advanced fibrosis regardless of the viral/metabolic trigger for the advanced fibrosis (COL1A1 ranges from approximately $1.3e+05$ to $7.4e+05$ ppb). The second pattern showed that ductular/metabolic markers exhibited significant differences in protein expression based on the etiology of the advanced fibrosis. For example, AKR1B10 had a much higher protein expression in individuals with NAFLD ($\sim 5.0e+06$ ppb) and mixed Hepatitis C and Alcoholic-related advanced fibrosis ($\sim 2.1e+06$ ppb), than in patients with viral hepatitis. A similar pattern is evident with SPP1 (Osteopontin) as it was also more highly expressed in NAFLD or mixed Hepatitis C and Alcoholic-related fibrotic patients than in pure viral hepatitis. A third example of variability in ductular/metabolic markers is demonstrated by KRT23, which also exhibits peak expression in mixed Alcoholic/non-alcoholic disease at $\sim 7.6e+04$ ppb.

These proteomic findings suggest that while the structural fibrotic backbone (COL1A1, THY1) may be common across patients, the intensity of the ductular and metabolic reaction (AKR1B10, KRT23, SPP1) is highly sensitive to the specific underlying etiology, with the strongest response observed in metabolic-associated fatty liver diseases.

DISCUSSION

This study aimed to define the transcriptional changes driving the transition from early to advanced liver fibrosis and to identify the specific cell types responsible for these signals. Our analysis of bulk RNA-sequencing data identified a distinct signature of 105 upregulated genes in advanced disease, dominated by ECM organization and epithelial development pathways.

Although the expression of collagen and genes involved in matrix remodeling was anticipated, cellular deconvolution of this signature with snRNA-seq revealed a key finding: that the leading transcriptional signal in advanced fibrosis tissue may not have been exclusively derived from HSCs, which are known for their matrix-producing abilities. This result confirms previous reports that related the extent of ductular reaction to the stage of fibrosis. It is well established that as fibrosis progresses, the cholangiocyte population becomes a mixture of senescent and proliferating cells that perpetuate inflammation [22–25]. By providing a gene expression portrait of the dominant signal and capturing ductular-derived EF signals through snRNA-seq, our data justify that the fibrogenic potential in late stage disease is not restricted to HSC but is amplified to a dramatic extent by the ductular niche as an epicenter of profibrogenic signaling molecules and matrix-producing cell mother cells.

Moreover, we here provide proteomic validation to show that although the fibrotic structure (collagen) is homogenously kept among patients, the epithelial-metabolic response differs substantially by patient's underlying etiology. This is consistent with recent evidence illustrating that the specific microenvironmental stressors of the disease opposed to a uniform scarring-dependent outcome intimately associate metabolism rewiring [26]. For example, despite some degree of commonality or minor differences in the spectrum of structural collagen variants among pathological situations, metabolic diversity of epithelial cells is extensive and frequently induced by distinct inflammatory and nutrient landscapes characteristic for a given disease [27]. This suggests that although the fibrotic structure is relatively unchanging, the epithelial metabolic response is dynamic and represents a target specific to disease. As a result, therapeutic options may extend past generic anti-fibrotic and target the unique metabolic weaknesses elicited by an etiology.

The finding of a strong ductular reaction signature (KRT23, EPCAM and SOX9) also contradict the simplistic hypothesis that bulk transcriptomic fibrosis signatures are simply reflections of HSC activation. Transcriptomic analysis historically has been associated with mesenchymal markers such as ACTA2 and COL1A1. In advanced fibrosis (METAVIR F3-F4), however, our data indicate that process of activation and proliferation of the biliary tree and hepatic progenitor cells may be the most statistically robust transcriptional event. It is in-line with histopathological data as the magnitude of ductular reaction strongly parallels disease severity and mortality. These activated epithelial cells are probably dynamic players in the fibrotic niche, releasing paracrine signals (like TGF- β and PDGF) that sustain HSC activation. Accordingly, therapies focusing exclusively on the downstream myofibroblasts are likely to overlook the upstream epithelial architecture that drives disease.

In large part, the ductular reaction's prominence has been substantiated by clinical evidence of how increased biliary proliferative activity affects patient outcomes. Studies have shown that fibrosis extent can be measured in a variety of ways; however, ductular reaction extent (using metrics such as CK7 [KRT7] & epithelial cell adhesion molecules) serves as an independent predictor for short-term mortality in alcoholic hepatitis [28, 29], and can predict survival from biliary atresia [30] through to hepatocellular carcinoma [31]. By defining a strong KRT23/EPCAM/SOX9 signature as the principal developmental event in F3-F4, our data provide molecular evidence to support these clinical observations because the severity of the fibrotic environment is not determined by collagen quantity alone, but also by the strength of pro-fibrogenic epithelial response to a given level of injury.

Our findings from the snRNA-seq study clarify a long-standing uncertainty regarding the use of bulk tissue analysis. For example, high levels of gene expression (e.g., SPP1 or ITGB6) often lead to confusion when interpreted using standard methodology. The use of snRNA-seq allowed us to map these genes specifically within the cholangiocyte cluster and establish their cellular source in advanced stage liver fibrosis. In addition to SPP1 being associated with metabolic dysfunction and bile duct obstruction [33], ITGB6 is associated with HSC and activated macrophage function during liver injury [34]. Since ITGB6 is an important activator of TGF- β , we suggest that the biliary epithelium serves as a localized control mechanism for

TGF- β activation on nearby fibroblasts to increase the deposition of scar tissue. The compartmentalization of cholangiocytes as the signal initiators and HSCs as the effectors creates a more accurate mechanical understanding of advanced fibrosis.

Fibrosis is known to have a structural basis, however, from our analysis of the subject's proteins, we also show that metabolic markers can separate patients into strata. Although the levels of COL1A1 and THY1 were comparable between patients with viral vs. metabolic etiologies, AKR1B10 had significant up-regulation only in the NAFLD & Alcoholic Liver Injury (ALI) populations [36-38]. AKR1B10 has two distinct functional pathways related to both detoxifying lipid peroxidation products as well as retinol metabolic conversion. The elevation of AKR1B10 is indicative of the fact that the liver is attempting to mitigate the damage caused by the different classes of toxins throughout metabolic disease through targeting particular toxic agents based on their source. Therefore, we propose that the implications for biomarker development from our data is the importance of a specific biomarker designated for either viral & or metabolic etiologies that cannot be fulfilled by a general fibrosis panel based purely on matrix markers that would demonstrate the fibrotic stage but not the metabolic context for the injurious process. In addition, the variable expression of SPP1 and KRT23 between the different etiologies of fibrosis provides further evidence that fibrosis does not represent a single molecular phenotype. While both exhibit similar morphological characteristics at the end of the process (i.e., scarring), the mechanisms to achieve the same result are quite divergent. During the progression of a metabolic syndrome patient, the ductular reaction is much more prevalent than that seen in a patient who has developed liver fibrosis as a result of syphilis. Clinical trials of anti-fibrotic drugs should be based on this heterogeneity to bring recognition to the fact that agents that affect metabolic pathways, will only have an effect in specific sub-populations with high target expression.

The limitation of this study is that it relies on previously established public datasets, thus not allowing for the opportunity to confirm findings with a new group. In addition, although centrifuged and concentrated single-cell RNA (snRNA) sequencing shows many cells, it may also miss rare or fragile cells compared to fully-resolved single-cell RNA sequencing (scRNA-Seq). Future work will be required to use spatial transcriptomics to identify the spatial location of these reactive cholangiocytes compared to activated hepatic stellate cells (HSCs) and confirm the proposed paracrine signaling mechanism in situ.

In conclusion, the transcriptional definition of advanced liver fibrosis involves a significant biliary epithelial cellular response that typically accompanies classical extracellular matrix (ECM) production. This cholangiocyte-derived signature represents both an indicator of fibrosis severity and a source of biliary-induced upstream signals associated with the development of fibrosis. By distinguishing between a generalized fibrosis 'backbone' and metabolic adaptations associated with specific disease etiologies, we provide a conceptual framework to enhance the biological classification and subgrouping of patients with chronic liver disease.

CONCLUSIONS

Advanced liver fibrosis can now be defined within a different transcriptional landscape according to this study. Stellate cells that produce matrix material and activated cholangiocytes may take part in orchestrating the progression of the disease. As part of this study, we were able to separate out universal structural factors related to the disease process from the etiology-specific metabolic adaptations seen in patients with advanced liver fibrosis. Based on these findings, current "one size fits all" therapeutic approaches are limited. We now recommend that the effective treatment of advanced liver fibrosis can only be accomplished through a shift to precision medicine. The therapeutic targets will be based on the individual patient's distinct disease etiology, and will take into account the corresponding metabolic and epithelial metabolic profile as they relate to viral and metabolic drivers of advanced liver fibrosis, respectively.

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