HHS Public Access

Author manuscript

Hepatology. Author manuscript; available in PMC 2022 December 19.

Published in final edited form as:

Hepatology. 2022 August ; 76(2): 372–386. doi:10.1002/hep.32326.

Progressive loss of HNF4α activity in chronic liver diseases in humans

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Abstract

Hepatocyte nuclear factor 4 alpha (HNF4α) is indispensable for hepatocyte differentiation and critical for maintaining liver health. Here we demonstrate that loss of HNF4α activity is crucial step in pathogenesis of chronic liver diseases that lead to development of hepatocellular carcinoma (HCC). We developed a HNF4α target gene signature which can accurately determine HNF4α activity and performed an exhaustive *in silico* analysis using hierarchical and K-means clustering, survival and rank order analysis of 30 independent datasets containing over 3500 individual samples. Association of changes in HNF4α activity to chronic liver disease progression of various etiologies including HCV and HBV induced liver cirrhosis, nonalcoholic fatty liver disease/ nonalcoholic steatohepatitis (NAFLD/NASH), and HCC were determined. The results revealed a stepwise reduction in HNF4α activity with each progressive stage of pathogenesis. Cluster analysis of liver cirrhosis gene expression datasets using the HNF4α signature showed that loss of HNF4α activity was associated with progression of Child-Pugh class, faster decompensation, incidence of HCC, and lower survival with and without HCC. A moderate decrease in HNF4α activity was observed in NAFLD from normal liver, but a further significant decline was observed in from NAFLD to NASH. In HCC, loss of HNF4α activity was associated with advanced disease, increased inflammatory changes, portal vein thrombosis and substantially lower survival. In conclusion, these data indicate that loss of HNF4α function is a common event in the pathogenesis of chronic liver diseases leading to HCC and is important from both diagnostic and therapeutic standpoints.

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Contribution of each authors:

S.G. conceived and conducted the study and helped to prepare the draft. I.H, D.R. and C.W. generated data used for analysis. S.W. reviewed drafts of the manuscript. U.A. conceived, conducted, funded the study from his grants and prepared the initial and final draft. All authors approved the draft.

Conflict of interest statement:

None of the authors have any conflict to declare

Introduction

Hepatocyte nuclear factor 4 alpha (HNF4α) is an orphan nuclear receptor expressed in the liver, kidney, pancreas and the gut (1–4). Its expression is highest in hepatocytes and it is rightly considered the master regulator of hepatic differentiation because it regulates genes involved in hepatocyte specific functions such as bile acid synthesis, drug metabolism, nutrient metabolism, urea clearance, and production of coagulation factors (3). Loss of HNF4α is embryonic lethal in mice and deletion of HNF4α in adult mice results in hepatomegaly, spontaneous proliferation and promotion of liver cancer(5–9). In humans, studies indicate that HNF4α expression is significantly reduced in alcoholic liver disease and in hepatocellular carcinoma (HCC)(10–13). Despite these studies, exact association of HNF4α function and chronic liver disease pathogenesis is not clear. For example, it is not known at which stage HNF4α expression and function declines in the pathogenic ark of steatosis, steatohepatitis, fibrosis, cirrhosis and HCC.

There is also an issue of HNF4α expression vs. its function. There is a possibility that HNF4α expression may not change but its function is downregulated (10, 12). We hypothesized that loss of HNF4α function correlates better with disease progression than does loss of its expression. To accurately determine HNF4α function in a tissue sample, we devised a HNF4α target gene signature using RNAseq data obtained from hepatocyte specific HNF4α knockout (HNF4α-KO) mice and further curated for human use. HNF4α gene transcription is regulated by two promoters (14). P1 promoter is active in adult liver and produces isoforms 1 and 2 leading to protein expression. P2 promoter is active during embryonic liver development and produces isoforms 7 and 8. At birth P2 promoter is switched off and P1 promoter is switched on. There are differences in P1 vs P2 (isoforms 1/2 vs 7/8) driven HNF4α target genes. Recent studies have revealed an HNF4α isoform switch in chronic liver diseases (14–16). We determined which isoform is involved in regulation of the signature genes to account for the isoform switch.

This 44 gene signature was used to determine loss of HNF4α function in various stages of chronic liver diseases of different etiologies that ultimately lead to HCC.

Two sets of studies were performed. Extensive *in silico* analyses was performed using 28 publicly available datasets from GEO containing a total of 3500 individual patient samples of normal, NAFLD, NASH, Child-Pugh class A Hep B-associated liver cirrhosis, early and advanced HCC and more. The results revealed that the novel HNF4α target gene signature is an excellent tool to study activity, which is readily affected by minute pathological changes in the liver.

Methods

Animals and sample collection

Generation of hepatocyte specific conditional HNF4α-KO mice and induction of HCCs in HNF4α-KO mice has been previously published (7). RNA isolated from WT and HNF4α-KO mouse livers was used for RNA-seq analysis. Further, RNA-Seq analysis was performed on diethylnitrosamine (DEN)-initiated liver tumors (three per genotype) obtained

from WT and hepatocyte-specific HNF4α-KO mice using methods previously described (7). All studies were performed in accordance with NIH guidelines and were approved by the Institutional Animal Care and Use Committee (IACUC) of KUMC.

Bioinformatic procedures

The detailed bioinformatic procedure to identify the HNF4α target gene signature, refining it for use in human samples, hierarchical clustering, survival analysis and rank analysis of data-sets are described in supplementary materials. To confirm the target gene signature, total hepatic mRNA from WT and HNF4α-KO mice were used as described before (7). Ct values of genes included in the HNF4α target gene signature were determined using a custom made TaqMan Gene Expression Assays containing species specific primers for each gene in the signature (Applied Biosystems, Foster City, CA). Each TaqMan array had 44 genes and 4 housekeeping internal controls in duplicates. The ΔCt values of signature genes were calculated by subtracting the geometric mean of Ct values from 4 housekeeping control genes (18S, GAPDH, HPRT, GUSB) from the Ct values of each signature gene.

Results

Development and validation of the HNF4α target gene signature

We hypothesized that HNF4α function rather than its expression declines with progression of HCC, and, that it might correlate with the HCC disease state. Because HNF4α is a transcription factor, we hypothesized that its target gene expression can be used as a measure of its activity. We identified an HNF4α target gene signature using a novel combinatorial bioinformatics approach as described in the methods section (Figure 1A). In the first step, we identified high fidelity HNF4α target genes (dataset 3) by comparing RNAseq data obtained from WT and HNF4α-KO mice (dataset 2) and RNAseq data obtained from diethylnitrosamine (DEN)-initiated HCC isolated from WT and HNF4α-KO mice (dataset 1). Dataset 3 contains the genes that change in the same direction (up or down) when HFN4α is lost in either normal livers or in liver cancer tissues. Next, we identified the genes directly regulated by HNF4α by comparing dataset 3 with previously published HNF4α ChIPseq data (dataset 4). Dataset 5 contained 286 genes that are affected in a similar manner when HNF4α is deleted and have a putative HNF4α binding site within 10kb from the transcription start site. These 286 genes could be considered the most high fidelity HNF4α direct target genes. Further, to identify a gene signature that can be used in human samples, we compared the 286 target genes from dataset 5 to GSE17548 (dataset 6), which contains gene expression data from 17 HCC and 20 cirrhosis samples. The comparison revealed 246 common genes (dataset 7), which were further curated using factor analysis-based filtering (see supplementary methods for details) to obtain the 44 gene HNF4α target gene signature (dataset 8). All datasets are provided in supplementary materials. We validated this gene signature using hepatic mRNA isolated from WT and HNF4α-KO mouse livers using TaqMan-based real time PCR analysis (Supplementary Figure 1). As expected, out of 44 genes, only two genes (*ITGA2* and *CYP39a1*) had increased expression in HNF4α-KO liver tissue, while the remaining 42 genes had significantly decreased expression. Next, we used this signature to perform extensive *in silico* analysis using a total of 28 independent global

gene expression datasets with a total number of 3500 samples obtained from TCGA and GEO databases (Supplementary table 1).

Isoform specificity of the HNF4α gene signature

It is known that HNF4α gene expression is regulated by two promoters, P2 promoter which is active during embryonic development and P1 promoter which is active in adults. Four types of HNF4α transcripts can be detected including 7 and 8 produced by P2 isoform and 1 and 2 produced by P1 isoform, which are dominant in adults. Recent studies indicate an HNF4α isoform switch during chronic liver disease and P2 promoter driven transcripts, isoform 7 and 8 can be found in adults. To determine whether the genes involved in the HFN4α gene signature can be regulated by one or both isoforms, we performed an additional bioinformatic analysis. Lambert et al. have recently published a study where each of the possible 12 isoforms were expressed in HCT199 cells and global gene expression was measured (17). We obtained these publicly available data (GSE125852) and performed a cluster analysis (Supplementary Figure 2). Our analysis showed that 65% of the signature genes are regulated in a similar fashion by isoforms 1, 2 (P1 driven adult isoforms), and 7 and 8 (P2 driven embryonic isoforms). These data indicate that majority of the genes in the signature are regulated by both HNF4α isoforms in the same manner increasing the confidence in its usage.

HNF4α activity declines with each progressive disease stage during HCC pathogenesis

We started our analysis with dataset GSE6764, which contains 75 samples of various stages of HCV-associated HCC pathogenesis from normal liver to very advanced HCC (Fig 2). An unsupervised hierarchical clustering using HNF4α target gene signature produced two distinct clusters (Fig. 2A). Cluster 1 (C1, outlined in red) contained 100% of the normal liver, low grade dysplasia, high grade dysplasia and cirrhosis samples. Additionally, it also contained 37% of very early HCC and 16% of advanced HCC samples (Fig 2B). Cluster 2 (C2, outlined in blue) contained 100% of early and very advanced HCC along with 62% of very early HCC and 84% of advanced HCC. These clusters were further divided into several subclusters which differentiated the samples according to their disease stage. For example, Cluster 1 contained a subcluster (SC3), which had only normal liver samples. Similarly, 8 out of the 10 (80%) very advanced HCC samples clustered together in subcluster 4 (SC4) within Cluster 2. These data demonstrate that HNF4α activity declines rapidly from cirrhosis to early HCC and further declined with HCC progression. It also demonstrates that the novel HNF4α target gene signature can differentiate between various pathological stages of chronic progressive liver diseases.

HNF4α function is a critical determinant of liver cirrhosis outcomes

We further dissected changes in HNF4α activity in the progression of liver cirrhosis, which is a common precursor in cancer pathogenesis in the liver initiated by varied etiologies.

K-means (K=2) cluster analysis was performed on gene expression data obtained from 216 HCV-associated early stage (Child-Pugh Class A) liver cirrhosis patients (GSE15654). This dataset is unique because these patients were prospectively followed for a median period of 10 years and several clinical outcomes such as cirrhosis stage progression, decompensation,

HCC occurrence and time to death are available. Cluster analysis separated the samples in two clusters based on the HNF4α target gene expression- Group 1 containing 85 samples with higher and Group 2 containing 131 samples with lower HNF4α target gene expression (Fig. 3A). Further analysis showed that in all clinical endpoints investigated patients with higher HNF4α activity had better outcomes. Patients with higher HNF4α activity showed significantly slower progression of liver cirrhosis (p= 0.00001, Fig. 3B) and had significantly less decompensation (p= 0.0035, Fig. 3C). Further, HCC occurrence was significantly lower in patients with better hepatic HNF4α function (p=0044, Fig. 3D). Initial HNF4α function was also associated with long term endpoints and patients with higher HNF4α activity had longer overall survival (p=0.0013, Fig. 3E), and survival without (p=0.034, Fig. 3F) and with HCC (p=0.013, Fig. 3G). These data indicate that loss of HNF4α activity is a critical step in progression of liver cirrhosis in either decompensation of the liver or development of HCC. Finally, loss of HNF4α activity is associated with shorter survival independent of whether the liver cirrhosis progresses to HCC or not.

Significant decline in HNF4α activity in NASH

In the next study, we determined change in HNF4α function during pathogenesis of NAFLD/NASH. The first study included dataset GSE33814, which contains a total of 44 samples including 13 normal, 19 steatosis and 12 steatohepatitis. The cluster analysis revealed three clusters (Fig. 4A). Cluster 1 (C1) had 10 samples with 80% (8/10) normal, 20% steatosis samples and showed highest HNF4α activity. Cluster 2 (C2) contained a total of 20 samples and had 20% normal (4/20), 60% steatosis (12/20) and 20% steatohepatitis (4/20) samples. Cluster 3 (C3), which showed the least HNF4α activity, had 14 samples with 57% steatohepatitis (8/14), 36% steatosis (5/14) and only 7% normal (1/14) samples. These data indicate that HNF4α activity declines moderately between normal and steatosis samples but a significant decline is observed between steatosis and steatohepatitis samples.

The second study included dataset GSE135251, which contains 206 NALFD/NASH samples in various stages of pathogenesis and 10 control samples. Along with the global transciptomics data, extensive clinical information was also available on these samples. To determine if HNF4α activity changes within the various stages of NALFD/NASH progression, we developed a novel rank order system (see supplementary methods for details) where patient samples from different pathological stages were rank ordered based on HNF4α function measured using the HNF4α target gene signature (Fig. 4B–E). Patients with NALFD and stage F0-F1 of NASH showed almost similar HNF4α activity, which rapidly declined in stages F2 through F4 of NASH (Fig. 4B). When rank order analysis was performed using NAS scores, patients with NAS scores 1 through 3 were showed only a moderate decline in HNF4α activity, patients with NAS score 4 through 6 showed a further significant decline but there was no difference between patients with NAS scores 5 and 6. However, patients with NAS scores 7 and 8, which grouped together, exhibited significantly lower HNF4α activity (Fig. 4C). This decline stage dependent decline in HNF4α activity was also observed when rank order analysis was performed using fibrosis scores over a scale of 0 to 4 (Fig. 4D). Finally, NASH patients were grouped in two major groups, showed significant decline in HNF4α activity between ‘early’ NASH and ‘advanced’ NASH.

Significant loss of HNF4α activity in tumors as compared to surrounding tissues in HCC.

In many cases, liver cirrhosis and NASH progress to HCC. We investigated changes in HNF4α activity in liver cancer using several datasets. First, we analyzed four separate datasets including GSE60502 (Fig. 5A), GSE14520-GLP571 (Fig. 5B), GSE14520-GLP3921 (Fig. 5C), and GSE64041 (Fig. 5D) containing HCC and paired surrounding non-tumor tissues from the same patient. Cluster analysis separated samples from GSE60502 containing 18 pairs, from GSE14520-GLP571 containing 19 pairs of tumor and surrounding NT tissue, and from GSE14520-GLP3921 containing 213 pairs of tumor and surrounding NT tissues in two groups, one with higher HNF4α activity and another with lower HNF4α activity. In all three analyses, the group containing surrounding NT tissue showed significantly higher HNF4α activity. Cluster analysis of GSE64041 containing 60 pairs of tumor and surrounding NT tissues along with 5 normal liver samples were separated in to three groups. Group 1a contained a total of 60 samples with all normal samples (5/5), 75% of all surrounding NT (45/60) and only 16% tumors (10/60) and showed the highest HNF4α activity. Group 1b containing a total of 20 samples which included 23% of all surrounding NT (14/60) and 10% of all tumors (6/60) and exhibited a moderate decline in HNF4α activity. Group 2 containing 45 samples showed the lowest HNF4α activity with only 2% surrounding NT (1/60) and 74% of all the tumors (44/60). These data indicate that surrounding NT maintains significantly higher HNF4α activity as compared to tumor tissues but there is some decline in HNF4α function in liver tissue around the tumor as compared to disease free normal liver.

Next, we extended these studies to determine changes in HNF4α in the liver which had HCC with portal vein tumor thrombus (PVT), which is an advanced stage of cancer with high mortality. Two datasets, GSE74656 containing 5 patients and GSE77509 containing 26 patients were used Each patient had three paired tissue samples including liver tumor, surrounding non-tumor tissue and the PVT (Supplementary Fig. 3). Cluster analysis using HNF4α gene signature produced three distinct clusters- group 1, group 2a and group 2b for both datasets. For dataset GSE74656, group 1 contained 100% (5/5) surrounding normal tissues (referred to as para-carcinoma), group 2a contained 80% of the tumors (5/5) and 40% of the PVT (2/5) samples and group 2b contained 20% of the tumors (1/5) and 60% of the PVT (3/5) samples. Cluster analysis of dataset GSE77509 obtained similar results with group 1 containing 95% of the surrounding normal (referred to as adjacent normal) tissue (19/20) and 5% of PVT (1/20), group 2a containing 5% of the adjacent normal (1/20), 75% of the tumors (15/20) and 55% of the PVT (11/20), and group 2b containing 25% of the tumors (5/20) and 40% of the PVT (8/20). In both analyses, group 1 which contained most of the surrounding normal tissue samples showed highest HNF4α activity, which declined significantly in group 2a containing mostly tumor samples. Group 2b containing mostly the PVT showed further significant decline in HNF4α activity as expected. These data indicate that decline in HNF4α function worsens in the livers that develop HCC with PVT.

Loss of HNF4α function correlate with lower survival in HCC.

To determine whether loss of HNF4α function is associated with lower survival, we used two datasets including The Cancer Genome Atlas (TCGA) containing 365 samples and dataset GSE20140 containing 82 samples (Fig. 6). Both datasets have patient survival

information in addition to global gene expression data on each patient. The gene expression data were subjected to cluster analysis using the HNF4α target gene signature and the resulting clusters were used for Kaplan-Meier (K-M) survival analysis. In the TCGA dataset, cluster analysis produced two clusters, group 1 with high HNF4α activity containing 76% of samples (279/365) and group 2 with lower HNF4α activity containing remaining 24% samples (86/365) (Fig. 6A). K-M plots revealed that patients in group 2 with lower HNF4α activity had significantly lower survival as compared to patients in group 1 with higher HNF4α activity (p=0.0001) (Fig. 6B). Similarly, samples in dataset GSE20140 were clustered in two groups. Group 1 with higher HNF4α activity had 48% samples (39/82) and group 2 with lower HNF4α activity had 52% samples (43/82) (Fig. 6C). K-M plots showed that patients in group 1 not only had significantly higher survival (p=0.091) (FIG. 6D) but also showed significantly lower rate of HCC recurrence (p=0.016) (Fig. 6E). These data clearly demonstrate that HCC patients with lower HNF4α function have worse disease outcomes.

The data that patients with higher HNF4α activity showed lower HCC recurrence were intriguing. We further investigated this using dataset GSE40873, which contains 49 noncancerous liver samples from HCC patients that met the Milan criteria and underwent partial hepatectomy treatment (Supplementary Fig. 4). In addition to global gene expression data on all patients, data on multicentric occurrence indicating recurrence of HCC in the same liver, are also available. The cluster analysis using HNF4α target gene expression produced two clusters including group 1 with higher HNF4α activity containing 53% samples (26/49) and group 2 with lower HNF4α activity containing 47% samples (23/49). K-M analysis showed that patients with higher HNF4α activity clustered in group 1 had a significantly lower multicentric occurrence (p=0.00096). These data further confirmed that HCC patients who have better HNF4α function even with the presence of HCC have a better outcome.

Rank order analysis reveals progressive decline in HNF4α activity during HCC progression.

From the standpoint of pathology, HCC is a diverse tumor type and is often found in the background of inflammation, fibrosis, fatty liver, cell death and cell proliferation. Our analysis, presented in Fig. 2 shows progressive loss of HNF4α activity from early HCC to advanced HCC. Various histopathological grading systems have been used to characterize this disease progression. In addition to HCC, cholangiocarcinoma (CCA) and mixed type HCC-CCA tumors also occur in the liver. We analyzed a total of 10 independent datasets containing over 1200 individual samples with several histopathological aspects on HCC progression using the rank order analysis (Fig. 7 and supplementary Fig. 5). Rank order analysis of samples from datasets GSE62232 containing 10 normal livers and 81 HCC tumor samples segregated by their Edmondson-Steiner (ES) grade revealed that HNF4α function is significantly lower in tumors as compared to normal tissues as expected (Fig 7A). Further, tumors with lower ES grade that correspond to early disease showed higher HNF4α activity as compared to tumors from higher ES grade indicating advanced disease (Normal > Grade I-II > Grade III-IV). Similar analysis of samples from dataset GSE84044 containing a total of 128 chronic hepatitis B infected patients separated using the Scheuer scoring system for

inflammation and fibrosis (Fig. 7B). Significant decline in HNF4α activity was observed in patients with 2–4 Scheuer score for inflammation as compared to 0 and 1 Scheuer score for inflammation. Further, patients with Scheuer score 0 and 1 for fibrosis showed higher HNF4α activity as compared to those with Scheuer score 2 and 3 for fibrosis. Patients with Scheuer score 4 for fibrosis, indicating most severe fibrosis, had significantly lower HNF4α activity as compared to all other groups (Fig. 7C).

We performed similar rank order analysis on eight other datasets, which corroborated the results obtained so far (Supplementary Fig. 5). HNF4α activity declined in surrounding non-tumor tissues compared to normal and further declined in HCC compared to surrounding non-tumor tissue (Supplementary Fig. 5A–B, GSE 62232 and GSE95698). Interestingly, there was no difference between male and female HCC samples, both of which showed significantly lower HNF4α activity compared to surrounding non-tumor tissue (Supplementary Fig. 5C, GSE76427). Analysis of datasets containing normal, HCC and cirrhosis (Supplementary Fig. 5G–H, GSE46444 and GSE25097) showed that HNF4α activity was highest in the normal tissues, and significantly declined in cirrhosis and HCC. Finally, analysis of datasets containing HCC, Fibrolamellar Hepatocellular Carcinoma (FLC), cholangiocarcinoma (CCC) and mixed tumor samples (Supplementary Fig. 5D–F, GSE57725, GSE57555 and GSE35306) showed higher HNF4α activity in HCC as compared to either FLC, or mixed tumors or CCC.

Discussion

The role of HNF4α in regulating hepatocyte differentiation and by extension liver function is well known (1, 2, 5). Over the years, studies have shown loss of HNF4α expression in chronic liver diseases, mainly in experimental rodent models and sporadically in patients. We and others have shown that deletion of HNF4α results in spontaneous proliferation in the liver, promotion of diethylnitrosamine (DEN)-induced liver cancers, and defective termination of liver regeneration resulting in 100% mortality(4, 7, 8, 18). Re-expression of HNF4α rescues mice subjected to partial hepatectomy by inducing differentiation, protects animals from cirrhosis, and decreases DEN-induced tumor formation (18–21). Loss of HNF4α has been documented in HCC (9, 13), in NASH (22, 23) and in alcoholic liver disease(10, 24). These studies thus indicate that decreased HNF4α is a characteristic of chronic liver diseases and that experimental depletion of HNF4α, by itself, can drive the progression to cirrhosis and HCC. Nonetheless, several unanswered questions remain. HNF4α loss takes places at which stage of the disease progression trajectory is not known. Further, it is not known whether loss of HNF4α protein expression is essential to promote disease or if simply loss of function, even when the protein is still expressed, leads to disease progression. In many cases, HNF4α expression does not change in the liver, even inside a tumor (15). We hypothesized that HNF4α function could be lost or decreased even when the protein is still expressed and determining HNF4α function at various stages of liver diseases that ultimately lead to HCC is essential to determine the role of HNF4α in chronic liver diseases. We hypothesized that because HNF4α is a transcription factor, expression of its target genes can be used as a measure of its activity within the liver tissue. To that end, we developed an HNF4α target gene signature that can accurately determine HNF4α activity.

We used a novel bioinformatics method to determine the 44 gene HNF4α target gene signature using RNAseq data from both mice and human samples. The genes included in the signature are high fidelity HNF4α targets that change based on HNF4α activity in normal livers as well as liver cancers. The signature genes are mainly positive targets of HNF4α, i.e., their expression is induced by HNF4α and include transporters (*Slc38a3, Slc27a5, Slc22a7*), enzymes involved in drug metabolism (*CYP39A1, CYP2D10, CYP2C29, CYP1A2*), nutrient metabolism (*SC5D, BAAT, Upb1*), complement activation (*C8A, C8G*) and nuclear receptors (*CAR and PPAR*). Out of the 44 genes, we consistently observed two genes upregulated in samples with low HNF4α activity including ITGA2 and CYP39A1, indicating these two could be negative targets of HNF4α. Overall, the HNF4α target gene signature reflects status of hepatocyte specific genes, which drive critical liver functions.

From bioinformatics standpoint, there are 12 possible isoforms of HNF4α. However, biologically only 4 transcripts are detectable which make two different proteins. HNF4α gene expression is regulated by two promoters, P1 and P2. During embryonic development P2 promoter is active and produces isoforms 7 and 8. At birth P2 promoter is switched offed and P1 promoter is turned on, which drives the expression of the adults 1 and 2 isoforms. Recent studies have shown that the P2 driven embryonic isoforms of HNF4α reappear in chronic liver diseases including alcoholic hepatitis and HCC (10, 14, 16). This raises the question whether our signature can account for the isoform switch. To address this, we performed cluster analysis on publicly available dataset GSE125852 (17), which consists of gene expression profile after ectopic expression of each individual isoform of HNF4α in cells. Our analysis showed that 65% of the signature genes are regulated similarly by P1 driven adult (1 and 2) and P2 driven embryonic (7 and 8) isoforms. This indicates that even if an isoform switch occurred, the signature expression can, for the most part, account for it. This also warrants development of a specific P2 driven signature in the future.

The goal of this study was the determine changes in HNF4α function during chronic liver diseases that ultimately lead to HCC such as hepatitis associated liver cirrhosis and NAFLD/NASH. Whereas using actual patient samples would have been interesting, the availability of well characterized patient samples in large numbers is a major limitation. To circumvent this and to use a large sample size to test our hypotheses, we performed an exhaustive *in silico* analysis using 28 separate publicly available datasets of global gene expression data on a total of 3500 patient samples. This provided us with a large enough sample size for robust statistical analysis. Cluster analysis, which allowed for segregation of patient samples into groups based on expression of HNF4α target genes, was highly effective because it provided both a visual and statistical method to determine changes in HNF4α activity within a particular sample set.

The major finding of this study is that HNF4α function is a highly sensitive index of disease progression in the liver. We observed decline in HNF4α function with each progressive stage of the disease from normal liver to very advanced HCC. The studies also identified inflection points in the disease progression trajectory where a major decline in HNF4α function occurs. A major decline in HNF4α activity occurs as livers progress from dysplasia to early cirrhosis and further from liver cirrhosis to early HCC. Similarly, we did not observe

significant loss of HNF4α function in steatosis as compared to normal livers but a major decline was identified in NASH as compared to steatosis. These data are consistent with previous findings in rodent models and in patient tissues in both liver cirrhosis and NAFLD/NASH (19, 22, 23). Further, analysis of several datasets of paired surrounding normal and HCC samples indicated that tumors always had significantly less HNF4α activity. However, rank analysis on datasets GSE76427 and GSE25097 (Fig.7) showed that the tissues surrounding the tumor may have significantly less HNF4α function as compared to a completely normal, disease free liver. These data indicate that HNF4α function is sensitive to the tumor microenvironment.

Further studies using samples of progressive stages of HCC clearly demonstrated a decline in HNF4α function as the early HCC tumors progress to more advanced disease. Rank analysis on Edmondson-Steiner gradation and Scheuer gradation indicated this progression could be associated with a proinflammatory environment in the tumors. The effect of inflammatory signaling on HNF4α expression and function is not completely clear but previous studies show that proinflammatory cytokines may decrease HNF4α activity, which is consistent with this observation (25, 26). Most importantly, patients who were able to maintain higher HNF4α activity had significantly higher survival. This was also observed in the case of liver cirrhosis, where a subset of patients with higher HNF4α function had better outcomes in all disease endpoints including decompensation, development of HCC and survival. These data further highlight the dominant and critical role of HNF4α in maintaining liver function.

These results lead to two main questions. First, can HNF4α target gene signature be used for disease prognosis? Second, is HNF4α a viable therapeutic target in preventing disease progression? Whereas both are real possibilities, many challenges remain. Our studies clearly demonstrate that this novel HNF4α target gene signature could be used to predict disease outcomes in cirrhosis as well as HCC. However, there are major challenges. Use of HNF4α signature as a prognostic tool will require excellent quality liver biopsies and measuring target gene expression in the RNA isolated from the small amount of tissue. With the improvement of radiological methods to diagnose and assess HCC progression, the number of liver biopsies performed has declined. The second challenge is to obtain good quality RNA in sufficient quantity to perform the gene expression analysis. Our preliminary studies indicate that qPCR-based gene expression analysis is extremely challenging due to limitations of both quantity and quality obtained from biopsy samples. Technologies such as NanoString which avoid cDNA conversion and amplification, may be useful but need careful study. Finally, our studies used existing datasets where cluster analysis grouped the samples based on either lower or higher HNF4α activity and allowed further comparison. Developing the target gene signature as a diagnostic test will require standardized normal control samples, which poses additional challenges. While these challenges are not unsurmountable, detailed translational studies are necessary.

Our studies clearly demonstrate that loss of HNF4α function is a critical step in progression of chronic liver diseases leading to liver cancers. These studies highlight HNF4α as a therapeutic target. Previous proof-of-concept rodent studies where HNF4α re-expression using viral vectors was done in liver cirrhosis and HCC samples (18–20, 26) support the

idea that restoring HNF4α activity will either reduce the speed of disease progression or completely prevent it. These studies warrant further efforts to identify small molecules or biologicals that can either maintain HNF4α function in a diseased tissue or re-stimulate HNF4α activity. Finding agents that can promote HNF4α function will have broad therapeutic application in several different chronic liver diseases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Grant support and Acknowledgments

These studies were supported by NIH-COBRE (P20 RR021940-03, P30 GM118247), NIEHS Toxicology Training Grant (T32 ES007079-34), NIH R01 DK0198414 and NIH R56 DK112768

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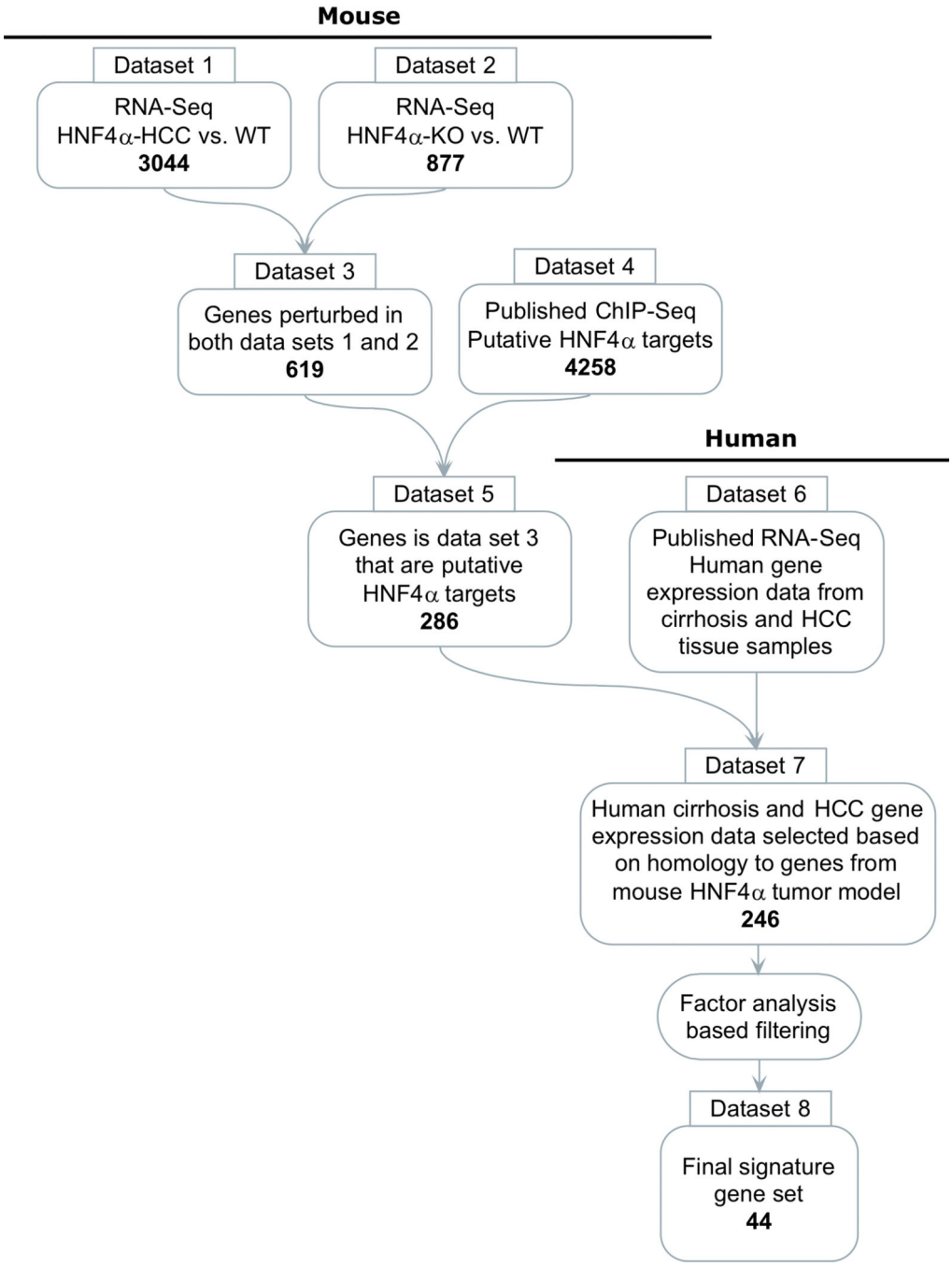

Figure 1.

Development and validation of HNF4α target gene signature to determine HNF4α function. Scheme shows the experimental design of the bioinformatics analysis to identify the HNF4α target gene signature.

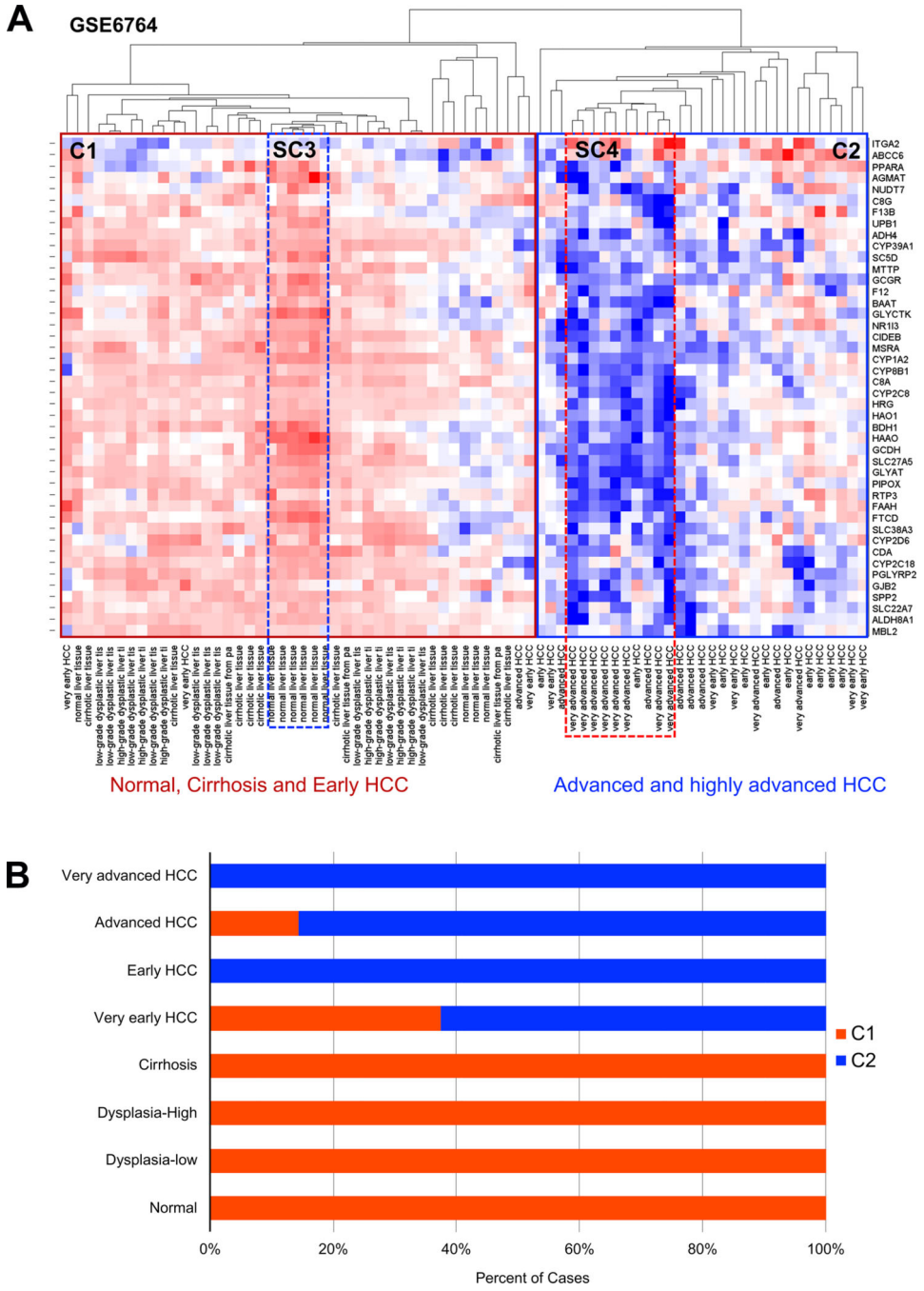

Figure 2.

Progressive loss of HNF4α function during various stages of HCC pathogenesis. (A) HNF4α target gene signature was used to perform cluster analysis on global gene expression data from GSE6764, a dataset containing 76 individual samples of normal livers and various pathological stages including low- and high-grade dysplasia, cirrhosis, early stage and advanced HCC. Cluster analysis yielded two main clusters. C1, outlined by solid red line, mainly included normal and cirrhotic samples, and C2, outlined by solid blue line, contained mainly advanced and highly advanced HCC. Further, two subclusters (SC3 and

SC4) were observed showing normal and very advanced HCC, respectively. White color in the heatmaps represents no change (no difference), blue color represents decreased (lower) expression and red represents increased (higher) expression. (B) Bar graph showing percent of each type of cases with either C1 or C2.

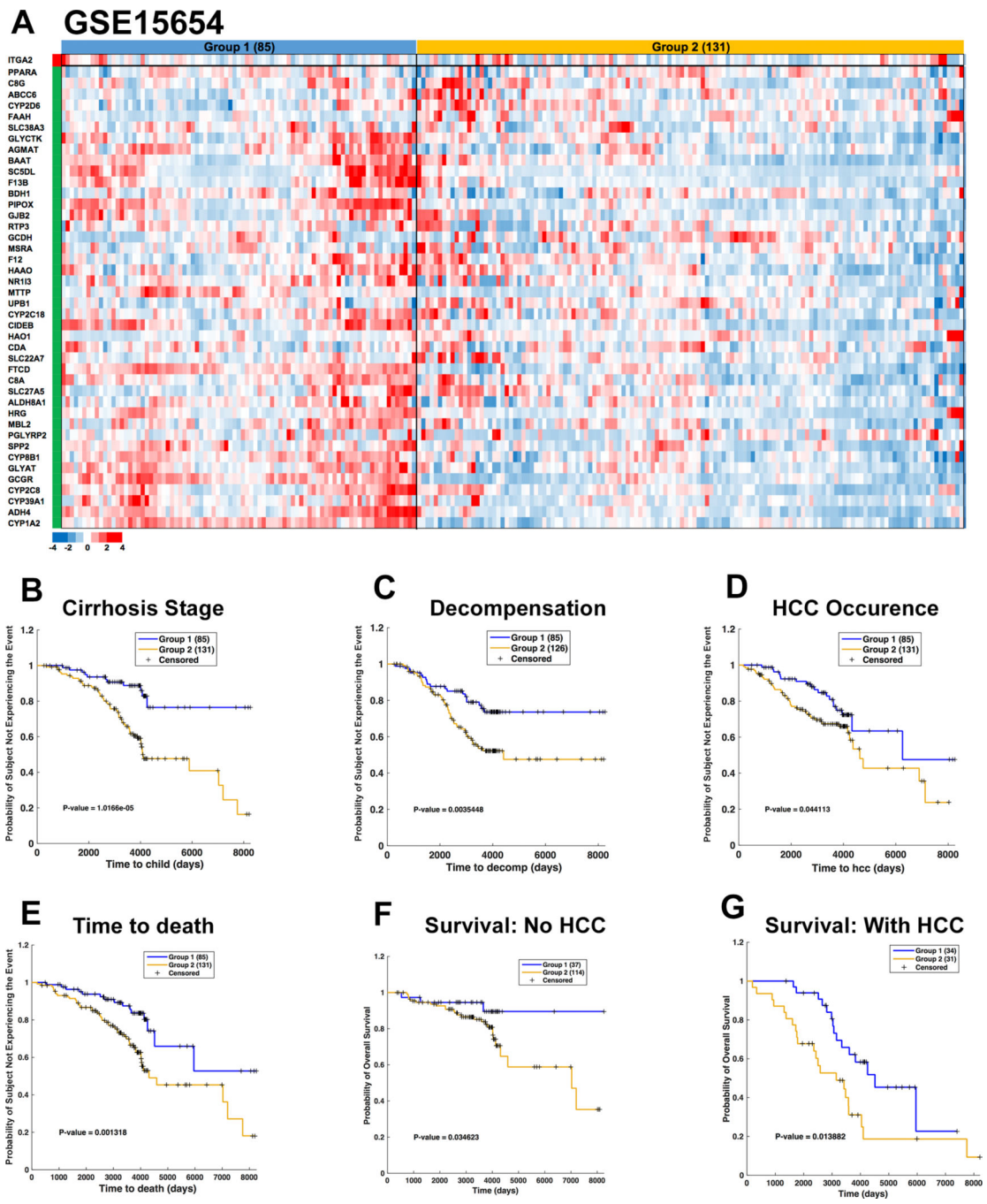

Figure 3.

Loss of HNF4α activity is a critical determinant of cirrhosis outcome. Cluster analysis was performed on dataset GSE15654 containing gene expression data from 216 early stage (Child-Pugh class A) liver cirrhosis samples. Further, Kaplan-Meier (K-M) survival estimation analysis was conducted to determine how each of the clusters performed for various endpoint. (A) Heatmap showing cluster analysis. White color in the heatmaps represents no change (no difference), blue color represents decreased (lower) expression and red represents increased (higher) expression. K-M plots were generated to determine

probability of the two groups (group 1 with high HNF4α activity and group 2 with low HNF4α activity) for reaching various clinical endpoints including (B) change (increase) in cirrhosis stage, (C) decompensation, (D) development of HCC, (E) overall survival, (F) survival in cases which did not develop HCC, and (G) survival in cases that developed HCC.

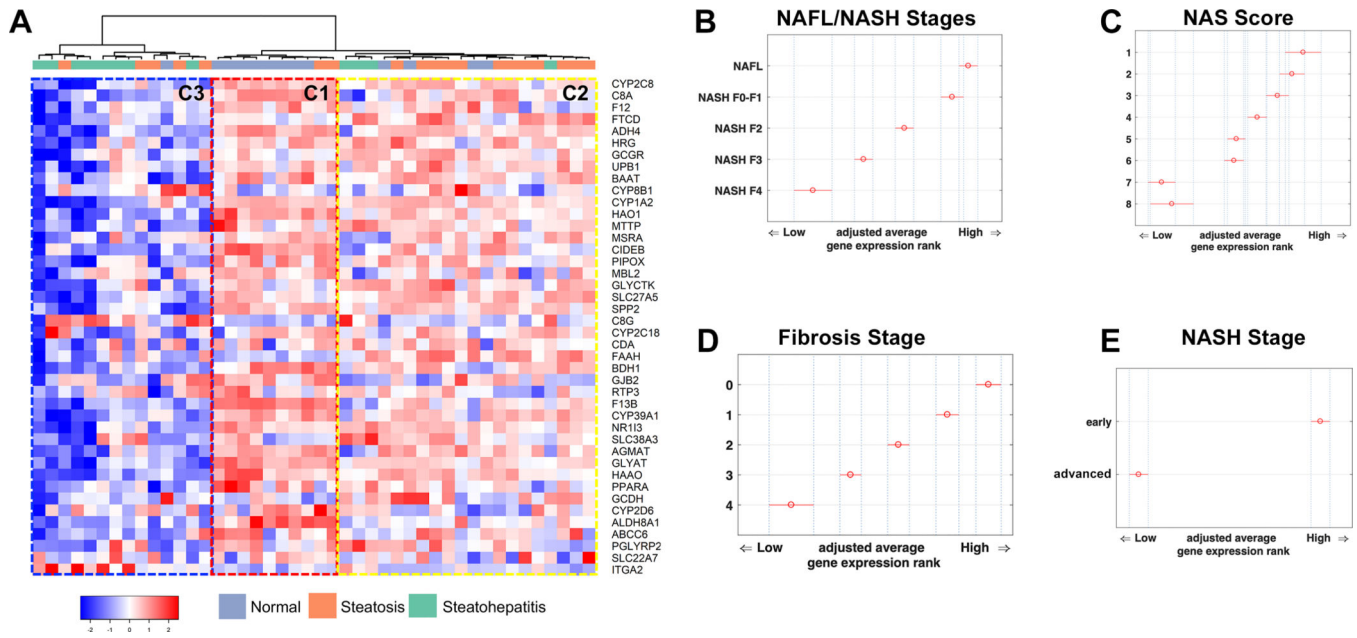

Figure 4.

Significant loss of HNF4α activity in nonalcoholic steatohepatitis (NASH). (A) Cluster analysis using the HNF4α target gene signature performed on dataset GSE 33814 containing 44 samples of normal, steatosis and steatohepatitis (NASH) patients revealed three clusters. Cluster 1 contained 80% normal and 20% steatosis samples, cluster 2 contained 20% normal, 60% steatosis and 20% NASH, and cluster 3 contained 1% normal, 35% steatosis and 57% NASH samples. Rank order analysis of samples from dataset GSE135251 containing 20 NAFLD/NASH and 10 control samples according to various clinical parameters including (B) NASH stage, (C) NAS score, (D) fibrosis score, and (E) early vs advanced stage of NASH.

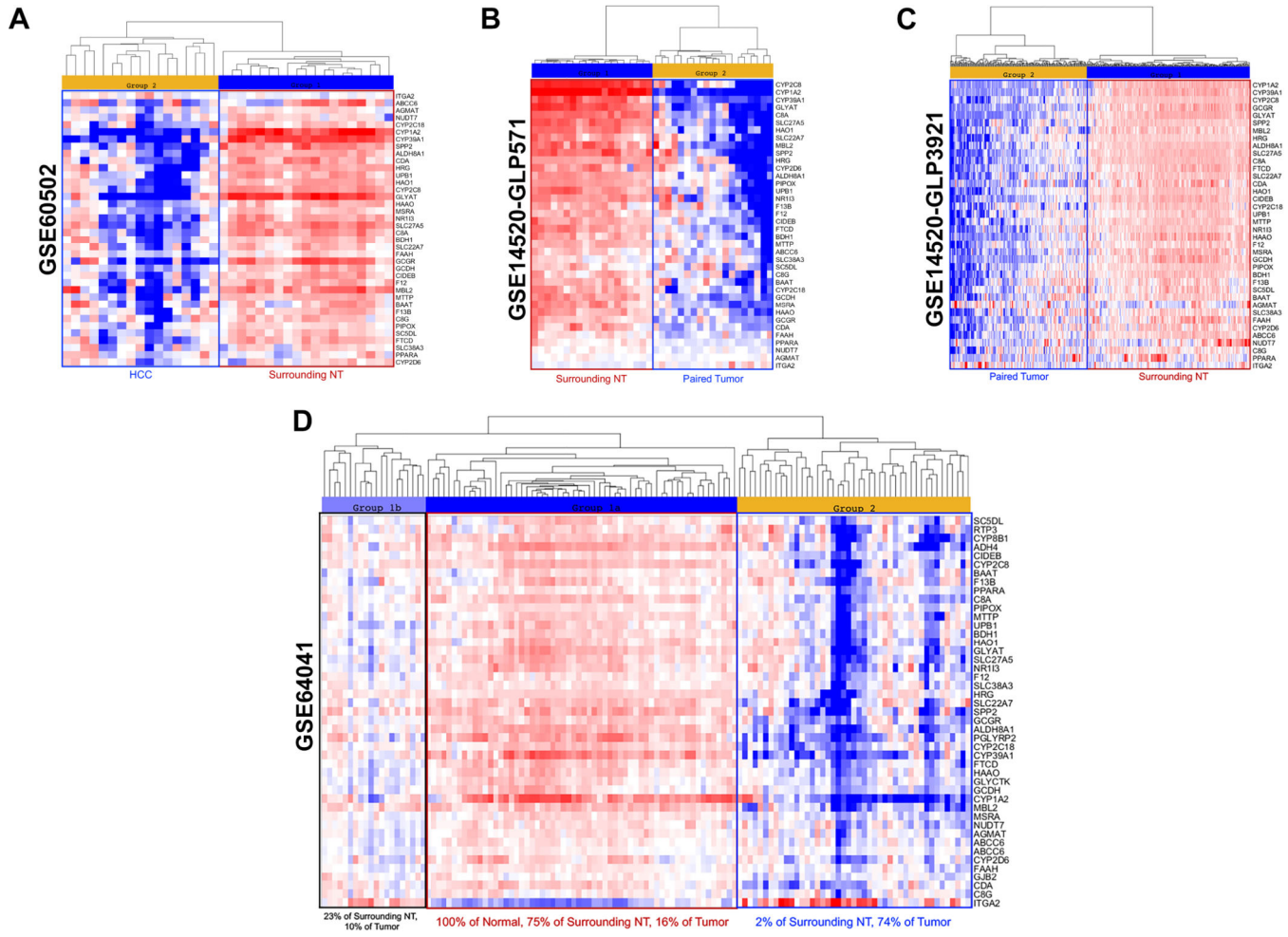

Figure 5.

HCC shows significant loss of HNF4α activity as compared to surrounding normal tissue. Cluster analysis using HNF4α target gene signature was performed on datasets containing gene expression data from paired tumor and surrounding non-tumor normal (surrounding NT) tissues. (A) dataset GSE60502 consisting of 36 samples (18 pairs), (B) dataset GSE14520-GLP571 containing 38 samples (19 pairs), (C) dataset GSE14520-GLP3921 containing 426 samples (213 pairs), (D) dataset GSE64041 containing 125 samples (60 pairs plus 5 normal tissues) of HCC and surrounding normal tissues. White color in the heatmaps represents no change (no difference), blue color represents decreased (lower) expression and red represents increased (higher) expression.

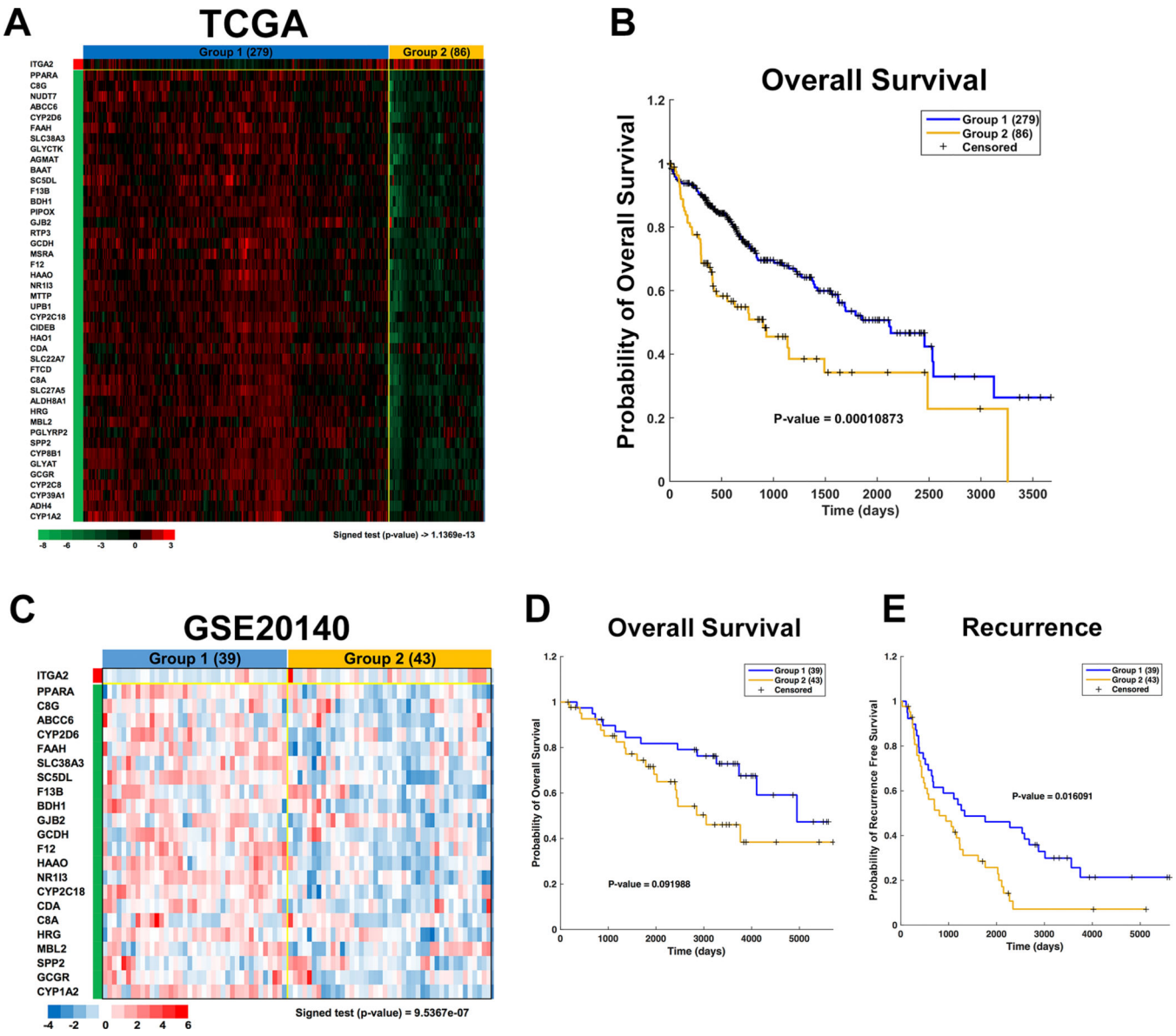

Figure 6.

HCC patients with higher HNF4α activity have higher survival. Cluster analysis using HNF4α target gene signature was performed on two datasets and accompanied survival data was used for Kaplan-Meier survival estimation analysis (K-M) (A), and (C) show heatmaps obtained from cluster analysis and (B), (D) show K-M plot of survival data from TCGA dataset and dataset GSE10143, respectively. (E) shows K-M analysis of HCC recurrence for dataset GSE10143. For (A) black color represents no change, red color represents increased (higher) gene expression and green color shows decreased (lower) gene expression. For (C) white color represents no change (no difference), blue color represents decreased (lower) expression and red represents increased (higher) expression.

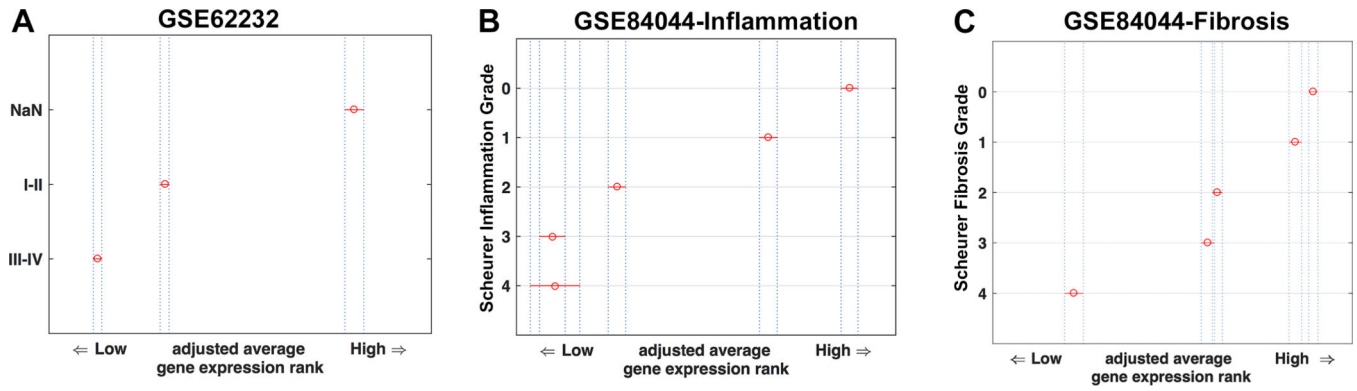

Figure 7.

Rank order analysis using HNF4α activity as measured by HNF4α target gene signature expression shows stepwise decline in HNF4α during chronic liver diseases. Rank order graphs of (A) dataset GSE62232 dataset containing 81 pairs of tumors and surrounding normal tissues separated by Edmondson Grades I to IV, (B) dataset GSE84044 dataset consisting of 124 HBV-associated liver cirrhosis separated by Scheurer score for inflammation, and (C) of the same data set separated by Scheurer score for fibrosis

Table 1:

List of genes in the HNF4α target gene signature

Symbol	Entrez Gene Name	NCBI Gene ID
ABCC6	ATP binding cassette subfamily C member 6	368
ADH4	alcohol dehydrogenase 4 (class II), pi polypeptide	127
AGMAT	agmatinase	79814
ALDH8A1	aldehyde dehydrogenase 8 family member A1	64577
BAAT	bile acid-CoA:amino acid N-acyltransferase	570
BDH1	3-hydroxybutyrate dehydrogenase 1	622
C8A	complement C8 alpha chain	731
C8G	complement C8 gamma chain	733
CDA	cytidine deaminase	978
CIDEB	cell death-inducing DFFA-like effector b	978
CYP1A2	cytochrome P450 family 1 subfamily A member 2	1544
CYP2C18	cytochrome P450 family 2 subfamily C member 18	1562
CYP2C8	cytochrome P450 family 2 subfamily C member 8	1558
CYP2D6	cytochrome P450 family 2 subfamily D member 6	1565
CYP39A1	cytochrome P450 family 39 subfamily A member 1	51302
CYP8B1	cytochrome P450 family 8 subfamily B member 1	1582
F12	coagulation factor XII	2161
F13B	coagulation factor XIII B chain	2165
FAAH	fatty acid amide hydrolase	2166
FTCD	formimidoyltransferase cyclodeaminase	10841
GCDH	glutaryl-CoA dehydrogenase	2639
GCGR	glucagon receptor	2642
GJB2	gap junction protein beta 2	2706
GLYAT	glycine-N-acyltransferase	10249
GLYCTK	glycerate kinase	132158
HAAO	3-hydroxyanthranilate 3,4-dioxygenase	23498
HAO1	hydroxyacid oxidase 1	54363
HRG	histidine rich glycoprotein	3273
ITGA2	integrin subunit alpha 2	3673
MBL2	mannose binding lectin 2	4153
MSRA	methionine sulfoxide reductase A	4482
MTTP	microsomal triglyceride transfer protein	4547
NR1I3	nuclear receptor subfamily 1 group I member 3	9970
NUDT7	nudix hydrolase 7	283927
PGLYRP2	peptidoglycan recognition protein 2	114770
PIPOX	pipecolic acid and sarcosine oxidase	51268
PPARA	peroxisome proliferator activated receptor alpha	5465
RTP3	receptor transporter protein 3	83597
SC5D	sterol-C5-desaturase	6309

Symbol	Entrez Gene Name	NCBI Gene ID
SLC22A7	solute carrier family 22 member 7	10864
SLC27A5	solute carrier family 27 member 5	10998
SLC38A3	solute carrier family 38 member 3	10991
SPP2	secreted phosphoprotein 2	6694
UPB1	beta-ureidopropionase 1	51733