

From cirrhosis to hepatocellular carcinoma: An investigation into hepatitis C viral oncogenesis

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Abstract

Background and Aim: Hepatitis C is a leading cause of chronic liver disease and hepatocellular carcinoma (HCC). Understanding the evolution and biology of HCC among HCV patients may lead to novel therapeutic avenues and risk stratification.

Material and Methods: Using meta-analysis platform STARGEO, we performed two separate meta-analyses as follows: 357 HCV-related HCC tumor samples with 220 adjacent non-tumor samples and 92 HCV-related cirrhotic liver samples with 53 healthy liver samples as a control. Then, we analyzed the signature in Ingenuity Pathway Analysis.

Results: HCV cirrhosis analysis demonstrated LPS/IL-1 mediated inhibition of RXR function, LXR/RXR activation, sirtuin signaling, IL-10 signaling and hepatic fibrosis/stellate cell activation as top canonical pathways. IL1 β , TNF, and TGF- β 1 were top upstream regulators. Cellular morphologic and signaling changes were noted through the up-regulation of RGS1/2, WNT receptor FZD7, the TGF- β 1-induced gap junction gene GJA1, and the zinc finger transcription factor repressor SNAI2. Apoptosis was inhibited through the down-regulation of OMA1. Metabolic dysfunction was noted through the down-regulation of SCLY and CBS. HCV-related HCC analysis showed FXR/RXR and LXR/RXR signaling, LPS/IL-1-mediated inhibition of RXR activation, and melatonin degradation as top canonical pathways.

Conclusion: Our results suggest that the genetic changes in the setting of chronic HCV infection predispose patients to developing HCC.

Keywords: HCC; HCV; STARGEO.

Introduction

Hepatitis C virus (HCV) is a leading cause of liver disease with chronic infection, potentially leading to cirrhosis in approximately 20–30% of the infective patients.^[1] HCV patients with cirrhosis are at hepatocellular carcinoma (HCC) development with an annual rate of \approx 3.5%.^[2] In

direct-acting antivirals (DAAs), era sustained virologic response (SVR) rates exceed 95% in HCV infection.^[3] However, the opioid epidemic has led to a rise in the incidence of HCV across the globe.^[4,5] In the era of DAAs, we are able to cure the majority of the HCV patients. HCC risk persists, especially in patients with advanced cirrhosis. First reports about the occurrence or recurrence of HCC after achieving SVR with DDAs were conflicting; some articles alleged potentially increased risk of HCC occurrence or recurrence,^[6,7] sequential reports refuted this argument.^[8,9] Moreover, new reports showed treatment with DAAs improved survival of HCV infected, even cirrhotic patients.^[3,10–12] Despite advancements in treatment in HCC, prognosis remains poor. Predicting which cirrhotic HCV patients will develop HCC remains a challenge. In the DAA cured patients, we still do not have long term data given that the INF free drugs only available since 2014. Factors that influence HCC *de-novo* occurrence or recurrence are being widely investigated. Male sex, diabetes, liver stiffness measurement and fibrosis-4 score were found independently associated with *de-novo* HCC, whereas diabetes was the only independent risk factor for recurrent HCC.^[13] Another study found out a lack of SVR and alpha-fetoprotein (AFP) as predictors of recurrent HCC.^[14] Understanding the evolution of liver fibrosis to HCC in HCV will pave the way for improved risk stratification and the development of novel therapeutic avenues. Nowadays, genes alterations in HCC following DAA treatment and pathological pathways from cirrhosis to HCC in HCV are being investigated.^[15] In this study, we aimed to characterize better the pathways involved in the oncogenesis of chronic HCV infection, and demonstrate the utility of crowd-sourced data and our STARGEO platform in the investigation of HCV-related HCC.^[16]

Materials and Methods

The National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO) is an open database of millions of biological samples from functional genomics experiments. The Search Tag Analyze Resource for GEO (STARGEO) platform allows for meta-analysis of genomic signatures of disease and tissue through tagging of biological samples from several experiments. More information on STARGEO and its functionality can be found in our previous paper.^[16] To study the different stages of progression from cirrhosis to HCC in HCV patients, we conducted a separate meta-analysis. For the meta-analysis of the cirrhotic stage of the disease, we tagged 92 HCV-related cirrhotic liver samples and tagged 53 healthy liver samples as a control from

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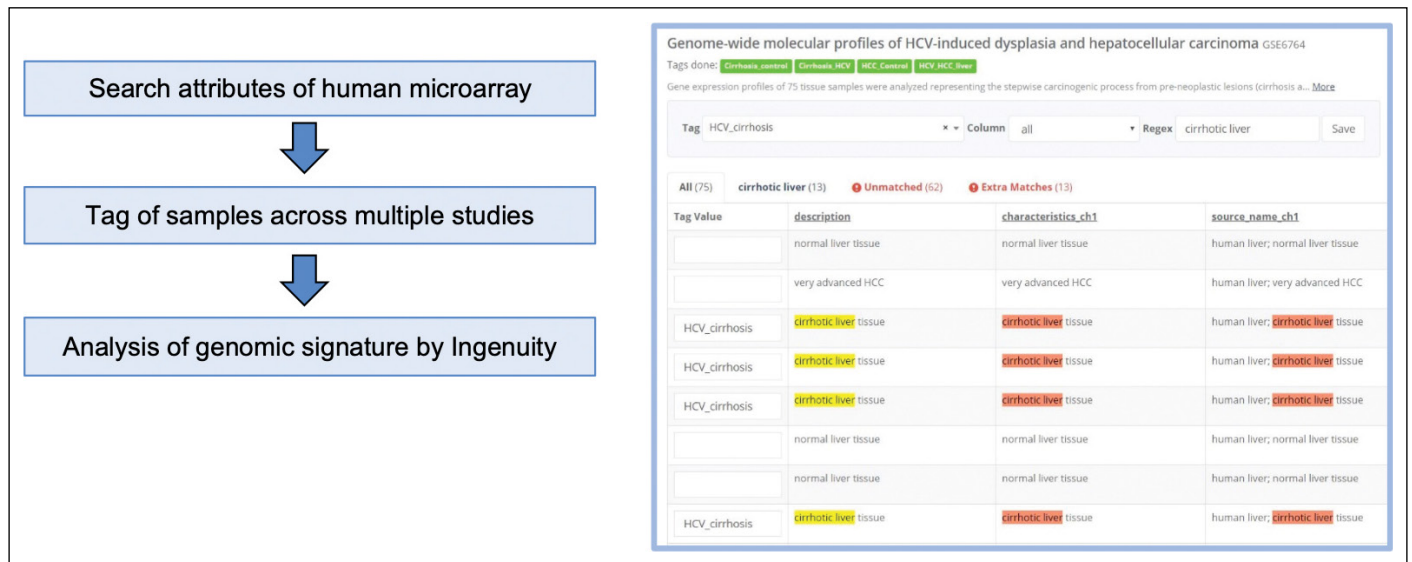


Figure 1. A screen capture of how to use STARGEO. Relevant experiments can be searched and samples from those experiments can be tagged using the regex function, as shown.

STARGEO: Search tag analyze resource for GEO; GEO: Gene expression omnibus.

Table 1. Summary of the list genes that are the most up-regulated and down-regulated in our meta-analysis of HCV-related cirrhosis and HCC. Experimental log ratios are shown

Top up-regulated				Top down-regulated			
HCV-Cirrhosis		HCV-HCC		HCV-Cirrhosis		HCV-HCC	
RGS1	0.834	DUXAP10	1.185	OMA1	-0.527	LINC01093	-2.258
PTGER4	0.581	NMRAL2P	1.130	SAA2	-0.313	CNDP1	-2.062
FZD7	0.552	CRNDE	1.125	LRIF1	-0.252	CLEC4G	-1.928
RGCC	0.524	AKRB10	1.090	CCDC69	-0.228	OIT3	-1.816
GJA1	0.416	CTHRC1	1.073	WWP1	-0.227	CLRN3	-1.814
DAB2	0.385	GPC3	1.072	SCLY	-0.211	TTC36	-1.763

a total of three series. For the meta-analysis of the HCC stage of the disease, we tagged 357 HCV-related HCC liver tumor samples and 220 adjacent non-tumor samples as a control from a total of 8 series (Fig. 1). Patients from these studies were not treated with DAAs. We were able to extract 1000s of genes for each of the meta-analyses conducted used STARGEO (see Table 1 for top up- and down-regulated genes). To evaluate this data, we analyzed the gene signatures from our meta-analyses in Ingenuity Pathway Analysis (IPA), restricting genes that showed statistical significance ($p < 0.05$) and an absolute experimental log ratio greater than 0.15 between conditions and control samples. These selected genes have been used for the next step analysis in IPA to elucidate the biological process, mechanisms of disease, and potential biomarkers and therapeutic targets that will be highlighted in our results and discussion section in this study.

IPA is based on the QIAGEN knowledge base and highlights biological pathways, drugs, and disease processes for OMICs data based on the most up-to-date literature. IPA contains millions of facts on the relationship between genes, disease processes, phenotype, drug activity, and more that can be searched for and highlighted in inputted genetic studies. These facts come from genomic experiments from several modal-

ities, including SNP and micro-RNA microarrays, RNA-sequencing, proteomic and metabolomic studies, chemical lists, and more. IPA allows us to dissect the complex biological networks that characterize genomic, metabolomic, and proteomic data.^[17] IPA allows us to take advantage of the novelty of our approach in using large scale data, and results from IPA analysis are demonstrated below.

All data analyzed were taken from Gene Expression Omnibus. There was no interaction or intervention with human subjects and no involvement with access with identifiable private patient information. As such, no IRB approval was necessary.

Results

HCV-Related Cirrhosis Analysis

We start with our analysis of HCV-related cirrhotic liver tissue. IPA analysis from our HCV-related cirrhosis study demonstrated LPS/IL-1 mediated inhibition of RXR (retinoid X receptor) function (p-value 4.38 E-06; z-score -1.633), LXR (liver X receptor)/RXR activation (p-value 5.10 E-06; z-score 0.707), sirtuin signaling (p-value 6.09 E-05; z-score -1.265), and IL-10 signaling (p-value 8.33 E-05; z-score NaN)

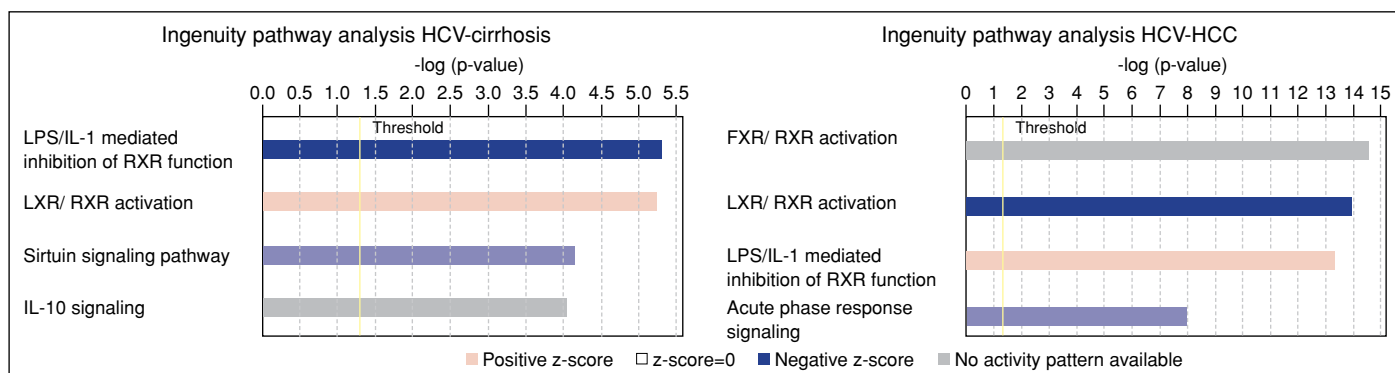


Figure 2. Top canonical pathways identified by Ingenuity Pathway Analysis from the HCV-related cirrhosis meta-analysis (above) and the HCV-related HCC meta-analysis. Z-score is illustrated in the legend.

(Fig. 2). From our HCV-related cirrhosis IPA analysis, we also identified interleukin 1-beta or IL1- β (p-value 8.03 E-11), transforming growth factor-beta or TGF- β (with predicted activation; p-value 3.49 E-76), tumor necrosis factor or TNF (with predicted activation; p-value 7.49 E-73), and interferon-gamma or IFN- γ (with predicted activation; p-value 2.33 E-49) as top upstream regulators. Our meta-analysis showed significant up and down-regulation of 1000s of genes between cirrhotic samples and healthy controls. The top up- and down-regulated genes have potential involvement in HCV infectivity, and oncogenesis is summarized in Table 1.

Among our most up-regulated genes were prostaglandin E2 receptor 4 or PTGER4 (p-value 0.0476, experimental log ratio 0.581). We also noted cell signaling pathways linked to oncogeneses, such as the up-regulation of genes involved in G protein-coupled signaling RGS1 (p-value 0.0412, experimental log ratio 0.834) and RGS2 (p-value 0.00, experimental log ratio 0.282), and in the pro-oncogenic pathway aryl hydrocarbon signaling, such as TIPARP (p-value 0.00, experimental log ratio 0.171). We found the up-regulation of the frizzled gene receptor FZD7. Additionally, we found up-regulation of RGCC, or regulator of cell cycle and known as RGC-32, (p-value 0.0188, experimental log ratio 0.524). We also noted up-regulation of genes gap junction alpha protein 1 or GJA1 (p-value 0.0172, experimental log ratio 0.416) and SNAI2 (p-value 0.00, experimental log ratio 0.321). We found the down-regulation of pyridoxal kinase (PDKX).

HCV-Related Hepatocellular Carcinoma Analysis

IPA analysis from our HCV-related HCC analysis demonstrated FXR (farnesoid X receptor)/RXR (p-value 1.41 E-10; z-score NaN) and LXR/RXR signaling (p-value 2.03 E-10; z-score -2.043), and LPS/IL1-mediated inhibition of RXR activation (p-value 2.12 E-12; z-score 1.633) as top canonical pathways. Our meta-analysis demonstrated the down-regulation of NR0B2 (p-value 4.51 E-4, experimental log ratio -0.204). From our HCV-related HCC IPA analysis, we also identified ERBB2 (with predicted activation, p-value 2.26 E-26), PPARA (p-value 7.16 E-26), ZBTB17 (p-value 2.77 E-23), CDKN1A (with predicted inhibition, p-value 1.08 E-21), and calcitriol (with predicted inhibition, p-value 1.24 E-21). We next investigated the relationship between calcitriol activity as an upstream regulator and genes relevant to tumor control or progression using IPA. IPA predicted that the decrease in calcitriol activity leads to downstream down-regulation of transcription factors and tumor suppressors CEPBD (p-value 1.27 E-4, experimental log ratio -0.190) and EGR1 (p-value 5.51 E-5, experimental log ratio

-0.446). We also noted a down-regulation of interferon regulatory factor 8 (IRF8; p-value 0.00216, experimental log ratio -0.232). The pro-apoptotic factor FAS (p-value 0.0196, experimental log ratio -0.151) was also down-regulated. Additionally, we found the down-regulation of the retinoid receptor RXRa (p-value 4.62 E-7, experimental log ratio -0.284). Lastly, we found up-regulation of pro-oncogenic transcription factor FOXM1^[18-20] (p-value 3.38 E-6, experimental log ratio 0.371) and oncogenic receptor tyrosine kinase KIT (p-value 0.0209, experimental log ratio 0.165).

The top two most up-regulated genes were the recently described oncogenic pseudogenes DUXAP10 (p-value 0.00312, experimental log ratio 1.18) and NMRAL2P (p-value 1.99 E-4, experimental log ratio 1.13). Additionally, we found up-regulation of the long non-coding RNA CRNDE (colorectal neoplasia differentially expression; p-value 4.41 E-4, experimental log ratio 0.320). We also found up-regulation of the gene collagen triple helix repeating containing 1 or CTHRC1 (p-value 4.05 E-5, experimental log ratio 0.261). Next, we wanted to investigate up-regulation of canonical beta-catenin/TCF targets given their role in cancer and found up-regulation of AXIN2 (p-value 3.59 E-4, experimental log ratio 0.383), LEF1 (p-value 1.98 E-8, experimental log ratio 0.677), and DKK1 (p-value 0.00345, experimental log ratio 0.230).

Discussion

HCV-Related Cirrhosis Analysis

IPA analysis from our HCV-related cirrhosis study demonstrated LPS/IL-1 mediated inhibition of RXR. The top canonical pathways highlighted reveal an overall activation of the LXR and RXR pathways at this stage of the disease. RXR and LXR are responsible for regulating lipid metabolism^[21] and may play a role in anti-HCV activity as HCV replication is linked to increased lipid metabolism.^[22] HCV core protein binds to RXR α and induces the activity of lipid metabolism enzymes, further supporting the role of lipid metabolism in HCV pathogenesis. Transcriptomic analysis of *in vitro* HCV replication has implicated activation of the “LPS/IL-1 mediated inhibition of RXR function” and inhibition of the “RXR/RXR” activation pathways in HCV activity.^[23] Given the role of lipid metabolism in HCV replication, this activity pattern may serve as an anti-viral response that is lost in the chronic infection phase of HCV, as would be seen in the cirrhotic liver samples we studied. Another potential role for these pathways is their potential role in cancer. Retinoids have a strong connection with HCC, with loss of retinoid activity being seen in HCC cell lines.^[22] Altered retinoid signaling and decreased stores of retinoid have been noted in both cir-

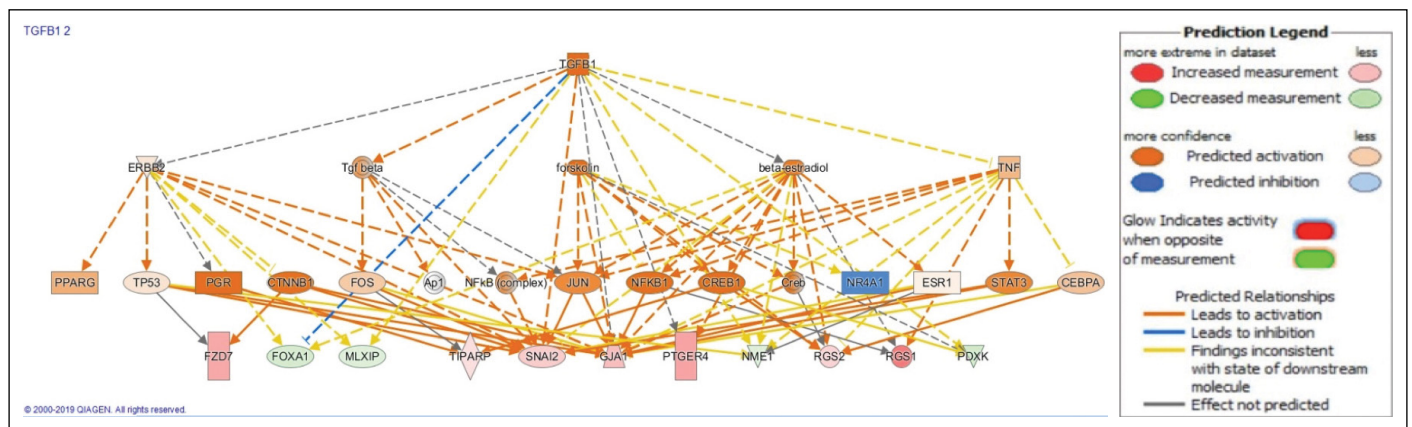


Figure 3. Ingenuity pathway analysis of several candidate genes with oncogenic and tumor suppressing properties downstream of TFGB1. Prediction legend below shows relations of genes.

rhosis and HCC.^[23] These past studies have motivated efforts in using acyclic retinoid in HCC prevention and treatment of liver diseases.^[24] Our results illustrate the overall agonism of RXR activity and may represent an anti-oncogenic response at the cirrhotic stage of the disease. RXR agonism may decrease over the course of the cirrhotic disease to the point where there is antagonism in RXR signaling at the point of HCC, as discussed below.

IL1- β , TGF- β , TNF, IFN- γ genes are top upstream regulators in HCV-related cirrhosis according to our analysis. All these genes have well-established roles in inflammation and are actively studied in the context of cirrhosis and liver disease. For example, HCV infection has been shown to induce IL1- β secretion in exposed Kupffer cells (resident liver macrophages) and, subsequently, drive hepatic inflammation.^[25] TGF- β has been shown to partake in all stages of disease progression from fibrosis to cirrhosis, and finally, to HCC.^[26] TGF- β contributes to liver fibrosis is through activation of hepatic stellate cells, differentiation of stellate cells to activated myofibroblasts, and apoptosis of hepatocytes.^[26] Although TGF- β has tumor-suppressive activity in the early stages of tumorigenesis, strong activation of TGF- β signaling is linked to tumor progression and may serve as a therapeutic target for HCC.^[27,28] The role of TNF in liver fibrosis and cirrhosis is not fully understood, but recent studies demonstrate its pivotal role.^[29] TNF signaling leads to hepatic stellate activation and survival and modulates other immune cells, including macrophages, dendritic cells, and B cells in contributing to liver fibrosis and cirrhosis.^[30,31] While the relationship between IFN- γ and stages of the disease have not been fully elucidated, there is a strong association between elevations of IFN- γ throughout all these stages.^[32] Additionally, IFN- γ has been shown to exacerbate liver damage in mouse models.^[33]

Our meta-analysis showed significant up-and down-regulation of 1000s of genes between cirrhotic samples and healthy controls. The top up- and down-regulated genes have potential involvement in HCV infectivity and oncogenesis (Table 1). We wanted to focus on our results and discussion on genes involved in liver fibrosis and progression of cirrhosis to HCC. One of our most up-regulated genes was prostaglandin E2 receptor 4 or PTGER4. Prostaglandin E2 has been shown to synergistically impair cytotoxic T cell function leading to the development of chronic infections, such as in HCV,^[34] and promotion of cancer, including obesity-related HCC.^[35] We also noted cell signaling pathways linked to oncogenesis, such as up-regulation of genes involved in G protein-coupled signaling RGS1, RGS2, and in the pro-oncogenic pathway aryl hydrocarbon signaling, such as TIPARP.^[36-39] We found the up-

regulation of the frizzled gene receptor FZD7, which causes increased Wnt/Beta-catenin activity and is associated with HCC.^[40] Soluble FZD7 inhibits Wnt signaling and sensitizes HCC cell lines towards doxorubicin.^[41] Additionally, we found up-regulation of RGC, or regulator of cell cycle and known as RGC-32, which is involved in cell proliferation, fibrosis, and cancer.^[42-46] Deficiency of RGC-32 can be protective from hepatic fibrosis, and potentially HCC, by limiting lipogenesis.^[47] We also noted the up-regulation of genes involved in cell adhesion and progression to malignancy, including gap junction alpha protein 1 or GJA1 and SNAI2. GJA1 has been associated with HCC^[48] and SNAI2 is a zinc finger transcriptional repressor which induces down-regulation of E-cadherin and promotes progression to HCC.^[49] Lastly, we found down-regulation of tumor-suppressive genes, including FOXA1 (p-value 0.00, experimental log ratio -0.143), OMAI (p-value 0.0298 E-7, experimental log ratio -0.527), NME1 (p-value 2.22 E-16, experimental log ratio -0.110 and PDKX (p-value 0.00, experimental log ratio -0.163). NME1 and FOXA1 have recently been shown to suppress HCC tumor cell viability through suppressing PI3K/Akt signaling.^[50,51] OMAI is a mitochondrial metalloprotease activated by Bax and Bak, leading to apoptosis and, its dysregulation has been linked to HCC.^[52] We found the down-regulation of PDKX required for vitamin B6 synthesis. Vitamin B6 has potent antioxidative effects and its deficiency may have a causative role in HCC.^[53] Since TGF- β is a top upstream regulator in our analysis and is implicated in liver disease progression, we illustrated its downstream effects on the genes discussed above (Fig. 3).

HCV-Related Hepatocellular Carcinoma Analysis

A fraction of patients with HCV-related cirrhosis will develop HCC. We conducted two separate meta-analyses as described above to illustrate the genetic drivers of this disease progression. The LXR/RXR activation pathway and LPS/IL-1 mediated inhibition of RXR activation are discussed above. As opposed to our HCV-related cirrhosis analysis, in this analysis, we see the overall activation of the RXR pathway. As discussed above, RXR agonism has an oncogenic effect and would be expected at this stage of the disease. FXR is a key regulator of bile acid synthesis and homeostasis.^[54,55] FXR also regulates the enterohepatic circulation of bile acids.^[56] Proper regulation of bile acids is paramount as it is toxic in excess, and improper FXR activity may cause the progression of inflammatory bowel disease, gallstones disease, liver fibrosis, and HCC.^[57-59] FXR activity may limit hepatic inflammation and, by extension, progression of liver disease. While IPA identified FXR sig-

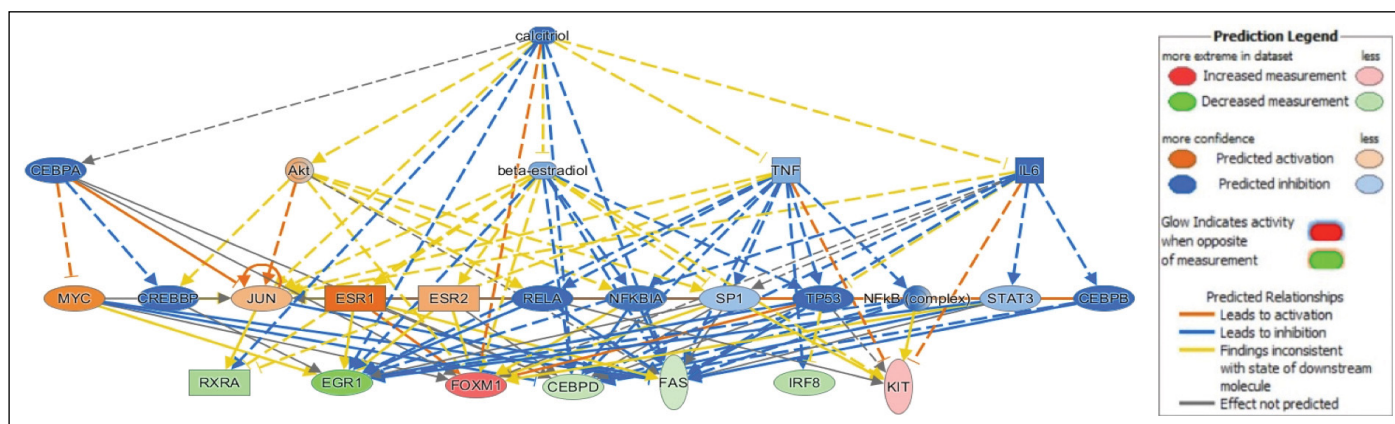


Figure 4. Ingenuity pathway analysis of several candidate genes with oncogenic and tumor suppressing properties downstream of calcitriol. Prediction legend below shows relations of genes.

naling as a top canonical pathway, it did not predict whether this pathway was activated or inhibited. We investigated the expression pattern of its target genes NR0B2, Abcb11, Ibbp, Osta, and Ostb. Our meta-analysis demonstrated down-regulation of NR0B2, but we did not have information on the other genes described, which may suggest inhibition of FXR signaling and subsequent promotion of tumor progression.

From our HCV-related HCC IPA analysis, we also identified ERBB2, PPARG, ZBTB17, CDKN1A, and calcitriol. ERBB2 has an underappreciated role in the context of HCC, but recent evidence links ERBB2 expression to HCC stage and tumor recurrence.^[60] PPARG, or peroxisome proliferator-activated receptor alpha, is a major regulator of lipid metabolism with evidence for anti-tumor activity and a potential role for regulation of HCC progression.^[61] ZBTB17 is a zinc finger protein that maintains MYC expression, a known contributor to HCC and hepatoblastoma.^[62] Using MYC activity, ZBTB17 may sustain tumor maintenance. CDKN1A, or p21, is a target of p53 and is linked to cell arrest in response to DNA damage.^[63] CDKN1A may inhibit HCC tumorigenesis.^[64] Our analysis predicts inhibition of its activity and, presumably, inhibition of cell arrest. Lastly, calcitriol has been shown to regulate the cell cycle, promote cell differentiation, and demonstrate anti-tumor activity within the tumor micro-environment.^[65] Calcitriol is being investigated as a potential therapy in HCC.^[66,67] We illustrated how the downstream effects of calcitriol inhibition on tumor suppressor and oncogene expression (Fig. 4). Downstream of calcitriol, we found down-regulation of transcription factors and tumor suppressors CEBPD^[68] and EGR1.^[69] We also noted IRF8, which functions as a tumor suppressor in some solid tumors.^[70,71] The pro-apoptotic factor FAS was also down-regulated. Additionally, we found down-regulation of the retinoid receptor RXRa, which would promote tumor progression as above. Lastly, we found the up-regulation of pro-oncogenic transcription factor FOXM1^[72-74] and oncogenic receptor tyrosine kinase KIT.

Next, we want to highlight several of the top up-regulated oncogenic genes. The top two most up-regulated genes were the recently described oncogenic pseudogenes DUXAP10 and NMRAL2P. DUXAP10 has been shown to promote the progression of non-small cell lung cancer (NSCLC) through interaction with oncogenic proteins and repression of the tumor-suppressive proteins.^[72] NMRAL2P was identified as a transcriptional target of the transcription factor Nrf2,^[73] which promotes the development of tumors.^[74] The silencing of NMRAL2P through CRISPR/Cas leads to inhibition of cancer cell growth and migration.^[73] While not previously studied in HCC, DUXAP10 and NMRAL2P may

have similar activity as described above. Additionally, we found up-regulation of the long non-coding RNA CRNDE (colorectal neoplasia differentially expression). CRNDE promotes cell survival, migration, and cancer cell proliferation in several cancer types.^[75] We also found the up-regulation of the gene CTHRC1. CTHRC1 enhances the adhesion and migratory activity of cancer cells and is linked with poor prognosis in HCC patients.^[76] When we investigated the up-regulation of canonical beta-catenin/TCF targets given their role in cancer and found up-regulation of AXIN2, LEF1, and DKK1. These target genes were not up-regulated in our HCV-related cirrhosis analysis and suggested a difference in beta-catenin activity as the disease progresses. Beta-catenin is expressed in the cell junction of hepatocytes and regulates cellular adhesion and communication.^[77] Alterations of this pathway are common in the development of HCC.^[77]

Conclusion

Our investigation illustrated the genetic changes in the setting of chronic HCV infection and cirrhosis that predispose patients to developing HCC. Some of these changes, such as LXR/FXR signaling and anti-tumor immune response, persist from the cirrhotic to the carcinoma stage. The other changes characterize what pathways and genes may drive progression from cirrhosis to HCC and may serve as potential therapeutic targets and biomarkers from liver biopsy analysis for patients at high risk for developing HCC. In the future, we plan on expanding our data set to investigate the immune micro-environment and to validate our results with patient samples.

Ethics Committee Approval: There was no interaction or intervention with human subjects and no involvement with access with identifiable private patient information. As such, no IRB approval was necessary.

Peer-review: Externally peer-reviewed.

Author Contributions: Concept – JA, HS, MP, DH, NA; Design – JA, HS, MP, DH, NA; Supervision – DH, AG, BS; Resource – JA, HS, MP, DH, NA; Materials – A, HS, MP, DH, NA; Data Collection and/or Processing – JA, HS, MP, DH, NA, MG, ND; Analysis and/or Interpretation – JA, HS, MP, DH, NA; Literature Search – JA, NA, DH, ND, MG, CS; Writing – JA, NA, ND, MG; Critical Reviews – DH, AG, BS.

Conflict of Interest: The authors have no conflict of interest to declare.

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