

[Open Peer Review on Qeios](#)

# Differential expression of key CmPn members distinguishes histological and immune subtypes of hepatic cancers

Johnathan Abou-Fade<sup>1</sup>, Victoria Reid<sup>1</sup>, Alexander Le<sup>1</sup>, Jun Zhang<sup>1</sup>

<sup>1</sup> Texas Tech University Health Sciences Center El Paso (TTUHSC)

**Funding:** No specific funding was received for this work.

**Potential competing interests:** No potential competing interests to declare.

## Abstract

Liver cancer, the sixth most diagnosed cancer worldwide is the third most common cause of cancer related deaths. The two most prevalent diagnosed subtypes include Hepatocellular carcinoma (HCC) and Cholangiocarcinoma (CCA) which comprise about 75% and 12-15% of all liver cancer cases, respectively. The liver is known to be a primary target and metabolic organ of the sex steroid hormone progesterone (PRG), which can induce its effects through either classic nuclear PRG receptors (nPRs), non-classic membrane PRG receptors (mPRs), or combined responses. In our previous studies we have demonstrated that the CCM signaling complex (CSC) couples both nPRs and mPRs to form the novel CmPn signaling network, which is involved in multiple cellular signaling pathways including angiogenesis and tumorigenesis of multiple cancers including breast cancers. Utilizing RNAseq data and Immunofluorescence approaches, we investigated the CmPn network to measure alterations to key cancer pathways during liver tumorigenesis by examining expression profiling for key CmPn members across multiple liver cancer subtypes, at both the transcriptional and translational levels. Our results demonstrated significant differential expression for multiple members of the CmPn signaling network including CCM1, PAQR7, PGRMC1, and nPRs, for both HCCs and CCAs, reinforcing the definitive roles of mPRs, nPRs, and CSC signaling during liver tumorigenesis. Therefore, we propose the future use of CmPn members' expression data as potential prognostic hepatic cancer biomarkers or biomarker signatures to not only distinguish between two major subtypes, HCCs and CCAs, but also to define some rare subtypes, such as undifferentiated pleomorphic sarcoma (UPS) and hepatic angiosarcoma (HAS), which may lead to hopeful new therapeutic strategies for hepatic cancers.

**Johnathan Abou-Fadel<sup>1</sup>, Victoria Reid<sup>1</sup>, Alexander Le<sup>1</sup>, Jun Zhang<sup>1\*</sup>**

<sup>1</sup> *Department of Molecular and Translational Medicine (MTM), Texas Tech University Health Science Center El Paso, El Paso, TX 79905 USA*

**\* All correspondence:**

*Jun Zhang, Sc.D., Ph.D.*

*Department of Molecular and Translational Medicine (MTM)*

*Texas Tech University Health Science Center El Paso*

*5001 El Paso Drive, El Paso, El Paso, TX 79905*

*Tel: (915) 215-4197, Email: [jun.zhang2000@gmail.com](mailto:jun.zhang2000@gmail.com)*

**Running Title:** CmPn network identifies novel prognostic biomarkers for hepatic cancers

**Keywords:** Liver cancer, liver cancer subtypes, immunological subtypes, classic nuclear progesterone receptors, non-classic membrane progesterone receptors, biomarkers, CCM signaling complex, tumorigenesis, progesterone.

**Abbreviations:** Hepatocellular Carcinoma (HCC), Cholangiocellular Carcinoma (CCA), CCM signaling complex (CSC), progesterone (PRG), nuclear Progesterone Receptors (nPRs), membrane Progesterone Receptors or Progestin and AdipoQ Receptors (mPRs/PAQRs), CSC-mPRs-PRG-nPRs (CmPn), CSC-mPRs-PRG (CmP), progesterone receptor membrane component 1/2 (PGRMC1/2), serum alpha-fetoprotein (AFP), immunofluorescence (IF), Hepatitis B virus (HBV), Hepatitis C virus (HCV), nonalcoholic fatty liver disease (NAFLD).

## Introduction

Liver cancer is the third most common cause of cancer related death and is the sixth most diagnosed cancer worldwide [1]. Hepatocellular carcinoma (HCC) and cholangiocarcinoma (CCA) are the leading primary liver cancers, comprising about 75% and 12-15% of all liver cancer cases, respectively [2]. 5-year survival rates for patients with liver cancer, in general, is ~5-30% [3], and more specifically, around 15% for HCC and 5-15% for CCA patients [4]. Patients diagnosed with HCC who receive early treatment are more likely to have longer recurrence-free periods and better overall survival rates compared to similar conditions in patients diagnosed with rarer subtypes of liver cancer [5]. Generally, the prognosis of HCC is poor as a result of their characteristic “chemoresistant” nature [6]. Studies have identified major prognostic factors that are associated with postoperative recurrence of HCCs to include tumor size, number of nodules, vascular invasion, tumor encapsulation, blood transfusion, high  $\alpha$ -fetoprotein (AFP) levels, and resection margin status [7]. Liver cancer is seen two to three times more often in males than females worldwide, which could be due to risk factors such as alcohol consumption, Hepatitis B virus (HBV), Hepatitis C virus (HCV), or nonalcoholic fatty liver disease (NAFLD) [8]. Though about 70% of CCA cases occur sporadically, risk factors such as HBV, HCV, infection-associated cirrhosis, diabetes, and obesity have recently been associated with increased CCA risks [9]. Although HCC and CCA are the most commonly diagnosed types of liver cancer, rarer liver cancer subtypes have worse prognosis and fewer successful treatment options available [10][11]. Low incidences of these rare hepatic cancers have made it difficult for in-depth analysis, as well as present challenges in early diagnosis before the cancer has metastasized [11]. Finally, since liver cancer is a male-dominant disease the role of Progesterone (PRG) signaling cascades is usually ignored.

The liver is known to be a primary target and metabolic organ of the sex steroid hormone PRG, and the possible role

that PRG plays in HCC progression has recently been explored [12][13][14][15]. PRG can act through classic, non-classic, or mixed responses, by binding either to classic nuclear PRG receptors (nPRs) or non-classic membrane PRG receptors (mPRs) [16]. It has been observed that the CSC, which is comprised of *KRIT1* (CCM1), *MGC4607* (CCM2) and *PDCD10* (CCM3), interacts with mPRs and nPRs to form the CSC-mPRs-PRG-nPRs (CmPn) signaling network under PRG actions [17][18]. The CmPn network has also been recognized to be involved in multiple cellular signaling pathways, most notably angiogenesis and the tumorigenesis of multiple types of cancers, including liver and breast cancers [17][19][20][21]. Within mPRs, there are 5/11 members that have been investigated for their involvement in angiogenesis and tumorigenesis of different cancer tissues: mPR $\alpha$  (PAQR5), mPR $\beta$  (PAQR6), mPR $\gamma$  (PAQR7), mPR $\delta$  (PAQR8) and mPR $\epsilon$  (PAQR9) [22][23]. This leads us to our current investigation on evaluating alterations to key members of the CmPn signaling network, among various hepatic cancers, for discovery of potential biomarkers for not only HCCs and CCAs, but for rarer subtypes as well.

Due to their suspected involvement in HCC, members of the CmPn network have been investigated as potential biomarkers. Down-regulation of progesterone receptor membrane component 1 (PGRMC1), a non-classical membrane progesterone receptor, has been associated with progression of HCC from tumor size G2 to G3 [24], and therefore, has been investigated as a possible prognostic biomarker for HCCs [25]. Previous studies have also shown that high *CCM3* gene expression promotes cell proliferation and metastasis of HCC, leading to poor prognosis [26]. Additionally, our foundational studies demonstrated altered expression of all three members of the CSC, along with PAQR7, during the early stages of tumorigenesis in liver cancer [20]. Currently, there is a lack of non-invasive diagnostic and prognostic measures for diagnosing hepatic cancer and differentiating the various subtypes which differ drastically in their histology and morphology. The current gold standard biomarker for diagnosing liver cancer is alpha-fetoprotein (AFP), which can also be used to distinguish between HCCs and CCAs [27]. AFP displays variable sensitivity and specificity, and therefore, is often used as a complementary biomarker, as high AFP levels can also be a sign of acute hepatitis, cirrhosis, or pregnancy [28]. The lack of sensitive/specific biomarkers used for the prognosis and diagnosis of liver cancer emphasizes the urgent need for identification of new potential biomarkers that can be utilized to not only diagnose liver cancer earlier, but also provide non-invasive prognostic methods to distinguish between liver cancer subtypes.

In our study, we profiled mRNA expression of key CmPn players including the CSC (*CCM1-3*), mPRs (*PAQR5-9*, *PGRMC1/2*) and nPRs (*PGR1/2*), along with the current “gold standard” liver biomarker AFP, across different liver tissue types and cancer types, in combination with clinical patient information. Additionally, we analyzed protein expression of key CmPn members including *CCM1/3*, *PAQR7/8*, *PGRMC1* and nPRs using immunofluorescence (IF) methods. Our results demonstrated potential for several key members of the CmPn network to be utilized as biomarkers for the diagnosis and prognosis of different liver cancer subtypes, at both the transcriptional and translational levels, through our comparative bioinformatics and IF approaches.

## Materials and Methods

## Bioinformatics analysis

Clinical profiling for hepatic cancers utilizing the NCI-GDC data portal. Utilizing the Genomic Data Commons (GDC) data portal from the National Cancer Institute (NCI), we assessed all available sociological/clinical/diagnostic data for patients diagnosed with liver (Hepatocellular carcinoma, HCC) or intrahepatic bile duct cancers (Cholangiocellular carcinoma, CCA) [29][30][31][32][33][34]. We performed preliminary general clinical analysis for HCC and CCA patients, which utilized 14 publicly available databases (Suppl. table 1) and repeated our clinical analysis for CmPn network associated clinical observations utilizing only 2 databases (Suppl. table 1) containing patient samples with differential expression data for any of the key CmPn players.

Differential expression profiling of key CmPn members, along with AFP, using microarray expression data. Expression analysis was performed as previously described [16]. Briefly, differential expression profiling was preliminarily performed utilizing the TCGA-TARGET-GTEX database, which is a unique database that contains two types of 'normal' tissues'; 1). "solid tissue normal" which are taken from normal tissue, near the tumor site and 2). 'normal healthy tissue' from individuals without cancer. This data originates from the UCSC RNAseq compendium, where TCGA, TARGET, and GTEx samples are re-analyzed (re-aligned to human hg38 reference genome and expressions are processed using RSEM and Kallisto methods) by the same RNA-seq pipeline, thereby eliminating batch effects. Additionally, the TCGA-PANCAN database was also used to validate results obtained using the TCGA-TARGET-GTEX database. Available sociological, pathological, and follow-up clinical data [35], for both HCC and CCA patients, was extracted from the TCGA-LIHC and TCGA-PANCAN databases and used during expression profiling to investigate differentially expressed genes among race, gender, family cancer history, vascular invasion, stemness scores across histological types, new tumor events (type and site), vital status, and residual tumor status [35].

Additionally, RNAseq data from the TCGA-TARGET-GTEX, as well as the TCGA-PANCAN databases, was used to investigate differential expression of key CmPn members, along with AFP, for both HCCs and CCAs, based on histological sample type (normal healthy tissue vs primary tumor samples). We then repeated expression profiling for each cancer type based on immune subtypes, in a pair-wise fashion. Finally, we evaluated expression profiling of key CmPn members, along with AFP, between HCCs and CCAs, to evaluate overlaps and differences in expression between these two cancer subtypes. Significant differential expression was performed using Xenabrowser with a cutoff p-value <0.05. Gene ontology, KEGG and reactome pathways enrichment analysis was performed from the RNAseq data using Enrichr. Significance was evaluated using a cutoff p-value <0.05 and FDR cutoff value <0.1.

## Immunofluorescence (IF)

Deparaffinization of paraffin-embedded tissue sections. IF processing of slides was performed as previously described [16]. Briefly, liver tissue microarray slides were purchased from US Biomax (Suppl. Table 3) and were baked at 60°C for 2 hrs. Once the slides cooled, the sections underwent 3 xylene washes at 5 min each, followed by sequential 3-min washes of 100, 95, 90, 80, and 70% ethanol and soaked in water before undergoing antigen retrieval.

**Antigen Retrieval.** Tissue sections were washed in 1X PBS 3 times, 3 min per wash before being permeabilized in PBS containing 0.2% Triton X-100 (wash buffer) for 10 min. Tissue slides were then submerged in 10mM sodium citrate buffer ( $\text{Na}_3\text{C}_6\text{H}_5\text{O}_7$ , pH 6.0) containing 0.01% Triton X-100. Tissues were maintained at 95-98°C for 30 min in citrate buffer and then set aside to cool at room temperature (RT).

**Blocking and Antibody Incubation.** Following antigen retrieval, slides were washed 3 times in wash buffer at 3 min per wash at RT. Tissues were then blocked using Pierce fast blocking buffer (Fisher) for 90 min at RT. A hydrophobic pen was used to draw a barrier around the tissue sections to conserve antibodies. All primary antibodies were diluted in wash buffer and Pierce fast blocking buffer (1:1). An initial 500ul was added to the tissues and left to incubate for 2 hrs at RT in the dark. Primary antibody was removed, and tissues were washed 3 times in wash buffer at 3 min per wash at RT. 400ul of secondary antibody, which was diluted in wash buffer and Pierce fast blocking buffer (1:1), was added to the slide and left to incubate for 2 hrs at RT. All antibodies used are listed in Suppl. Table 3. Tissues were washed 3 times in wash buffer at 3 min per wash at RT before continuing to the mounting/sealing step.

**Nuclear Staining and Mounting/Sealing.** Tissue sections were stained with DAPI by adding two drops of mounting media containing DAPI directly to the sections. Tissues were left to rest O/N at 4°C in the dark to allow efficient staining of DAPI. Slides were sealed with nail polish the next day and allowed time to dry before imaging.

**Imaging and Quantification.** IF imaging/quantification of microarray slides was performed as previously described<sup>[16]</sup>. Briefly, imaging was performed utilizing a Nikon Eclipse Ti confocal microscope using 10X, 20X and 60X objective lenses. Nikon Elements Analysis software, equipped on the Nikon microscope, was used to automatically quantify protein abundance in the tissues. Bias was avoided by maintaining thresholding values across all images and to dismiss low and high outliers. IF images were quantified for CCM1 and PGRMC1 using 488nm wavelength channel, while images for CCM3, PAQR7/8, nPRs, and AFP were quantified using the 555nm channel.

## Prognostic outcomes for key CmPn members using HCC patient samples

**Construction of Kaplan-Meier (KM) survival curves to determine prognostic effects.** Publicly available microarray data (22,277 probes) from 364 liver cancer patients (only available for HCCs due to small sample size for CCAs) was analyzed using KMplotter<sup>[36]</sup> to integrate gene expression and clinical data simultaneously. To ensure patients in the database reflected cohorts seen in the everyday clinical practice, we filtered the patient data by only selecting cohort data similar to SEER published prevalence<sup>[36]</sup>. Additionally, publicly available microarray data from 374 liver cancer patients (again, only available for HCCs due to small sample size for CCAs) was also assessed from TCGA to integrate gene expression and clinical data simultaneously<sup>[35]</sup> to confirm the initial analysis performed using KMplotter. Logrank P-values, hazard ratios and 95% confidence intervals were calculated by the software<sup>[36]</sup>.

## Statistical Analysis

**For IF analysis,** One-way analysis of variance (ANOVA) or two-way ANOVA (for co-stained sections) were used to detect

differences in the mean abundance of proteins among the various liver tissue types with Holm-Sidak's multiple comparisons correction. Welch's *t*-test was used to detect the differences in the mean values among two comparing groups. For Bioinformatics Analysis, Welch's *t*-test was also used to detect the differences in the mean values among two comparing groups, while one-way ANOVA was used to detect the differences in the mean values among more than two groups. All IF graphs/plots/charts were constructed and produced using GraphPad Prism 9.3.1, while bioinformatics graphs/plots/charts were produced using either the Xena, KMplotter or NCI-GDC built-in analysis software.

## Results

### Clinical profiling of hepatic cancers with altered CmPn expression

Given our recent findings of the potential role of the CmPn signaling network in tumorigenesis of multiple types of cancers [16][20][21][37][38][39], we began our investigation by assessing general clinical information for hepatic cancers utilizing the Genomic Data Commons (GDC) data portal from the National Cancer Institute (NCI). We then compared these results to filtered data only analyzing patient samples with differential expression for any key CmPn players (Fig. 1, Suppl. Table 1). By filtering the NCI-GDC data to look only at patients with differential CmPn expression, we observed that approximately 80% of hepatic cancer patients with altered expression of key CmPn members had a life expectancy of ~9 years, compared to ~10 years for hepatic cancer patients without modified CmPn expression (Figs. 1A-1 & 1A-5, left panels). It was also noted that patients with CmPn alterations were diagnosed later in life ~61 years old, compared to ~58 years for hepatic cancer patients without modified CmPn expression (Figs. 1A-1 & 1A-5, right panels). Both year of diagnosis (Figs. 1A-2 & 1A-6) and year of death analyses (Figs. 1A-4 & 1A-8) were similar, indicating that regardless of differential expression of key CmPn members, there has been an exponential increase in both categories for hepatic cancer patients. Interestingly, when evaluating tissue of origin (Figs. 1A-3 & 1A-7), there was a noticeable increase in the number of cases originating in the intrahepatic bile ducts for patients with differential expression of CmPn members (~12%) compared to patients without CmPn alterations (~3%) .

We additionally performed socioeconomic status (SES) and diagnostic comparative profiling utilizing clinical data for patient samples without (Fig. 1B1-1B5) or with differential expression for key CmPn players (Fig. 1B6-1B9). Our analysis revealed that the majority of patients diagnosed with hepatic cancers were not Hispanic/Latino, irrespective of CmPn members' expression (Figs. 1B-1 & 1B-6, left panels). The percentage of patients among the four primary racial categories between our two groups were relatively similar, although with a higher number of Asian patients (~40%) with altered CmPn expression data (Fig. 1B-6, right panel) compared to patients without CmPn alterations (~14%; Fig. 1B-1, right panel). Gender stratification of patients illustrated that hepatic cancer is dominant in males (Figs. 1B-2 & 1B-7) regardless of altered expression of key CmPn members. Interestingly, an 11% increase was observed in patients with prior malignancy with differential expression of CmPn genes (Fig. 1B-8) compared to those without (Fig. 1B-3). Additionally, when we examined primary diagnosis status, we saw that nearly half of cases were identified as either CCA or HCC, while <1% were recognized as either combined hepatocholangiocarcinoma (cHCC-CCA) or clear cell adenocarcinoma (CCC)



(Fig. 1B-4) for patients without CmPn alterations. Interestingly, when we repeated the analysis for patients with altered CmPn expression, we observed a switch in the primary diagnosis classifications in which now ~80% were diagnosed with HCC and only ~10% with CCA (Fig. 1B-9), while cHCC-CCA and CCC remained the least diagnosed subtypes. Finally, we conducted an assessment on general tumor classification data for patients without CmPn expression (not enough data for altered CmPn expression analysis) and observed more hepatic cancer cases that began as primary cancers rather than a result of metastasis from another organ (Fig. 1B-5).

## Altered expression of CmPn genes across clinical tumors in hepatic cancers suggest their involvement in liver cancer tumorigenesis

Based on our preliminary clinical analysis of altered SES and diagnostic profiling between patients with and without differential expression of key CmPn players, we next evaluated expression of CmPn players in liver tissues using publicly available RNAseq data from The Cancer Genome Atlas (TCGA). To evaluate differential expression of key CmPn players and their potential role as prognostic biomarkers, we also profiled AFP, an established liver cancer biomarker, to compare with our CmPn profiling analysis. For our first analysis, we utilized two databases in TCGA containing two categories of 'normal' tissues; "solid tissue normal" which are normal tissue sections taken adjacent to primary tumor tissue and "normal healthy tissue" taken from healthy individuals with no diagnosis of cancer. We observed significant differential expression patterns, in both HCCs and CCAs, for almost all CmPn players (except *PGRMC2* in CCAs), along with *AFP*, in our preliminary analysis (Fig. 2A-1 & 2A-2). Next, we repeated expression profiling using a separate database in TCGA, based on tissue type (normal healthy tissue unavailable in this database), confirming several of our previous observations. We confirmed significant differential expression patterns for *CCM2*, *PAQR6/8*, *PGRMC1/2*, *nPRs*, along with *AFP*, for HCCs (Fig. 2B-1), while analysis of CCAs (recurrent tumor data unavailable) confirmed significant differential expression patterns for almost all CmPn players (except *CCM1/PAQR7*), along with *AFP* (Fig. 2B-2).

To further validate the role of the CmPn network during liver cancer tumorigenesis, we analyzed differentially expressed genes (DEGs) in a pair-wise fashion, comparing normal healthy liver tissue to HCC primary tumor tissues, using publicly available RNAseq data. After analyzing DEGs between 110 normal healthy liver tissues and 369 HCC primary tumor tissues (Suppl. Table 2A), we observed down-regulation of *CCM1/2*, *PAQR6*, and *nPRs* in HCC primary tumor tissues, while all other CmPn players were up-regulated, along with *AFP* (Fig. 2C-1). We then utilized all DEGs to perform pathway enrichment analysis to display the top 15 affected biological processes, molecular functions, and KEGG pathways between HCC primary tumor tissues and normal healthy liver tissues (Fig. 2C-2 and Suppl. Table 2A). Our results illustrated that up regulated DEGs in HCC primary tumors significantly affected cell cycle regulation, protein binding, ATP-dependent activity, histone phosphorylation and cancer signaling pathways including DNA replication, spliceosomes/lysosomes, pancreatic cancer, and hormone signaling pathways including Oocyte meiosis and Progesterone-mediated oocyte maturation (Fig. 2C-2, left panels and Suppl. Table 2A). Down-regulated DEGs significantly affected actin filament-based processes, protein digestion and absorption (also seen in a recent analysis of HCC tumors using 4 separate independent databases) <sup>[40]</sup> and pancreatic secretion (Fig. 2C-2, right panels and Suppl. Table 2A). Interestingly, dysfunction of pancreatic secretion has been reported as a metabolic change resulting from liver

cancer [41][42]. Several important cancer signaling pathways were also observed to be affected (with significant p-values, but FDR>0.1) including P53 signaling, viral carcinogenesis, colorectal cancer, base excision repair, and the Fanconi anemia pathway (Fig. 2C-2, lower left panel and Suppl. Table 2A).

We repeated DEG analysis this time comparing 110 normal healthy liver tissues to 36 CCA primary tumor tissues, which demonstrated more overall down-regulated CmPn genes in CCA primary tumors, compared to our HCC analysis (Fig. 2D-1 and Suppl. Table 2B). We observed down regulation of *CCM1/2* (down in HCC), *PAQR9* (up in HCC), *PGRMC1/2* (up in HCC), *nPRs* (down in HCC) and *AFP* (up in HCC) in CCA primary tumors. Interestingly, *CCM3* and *PAQR5/7/8* were up regulated in CCAs (Fig. 2D-1 and Suppl. Table 2B) which were also up regulated in our HCC analysis. Utilizing DEGs from CCAs we performed pathway enrichment analysis and observed that up-regulated DEGs in CCA primary tumors were significantly enriched in mainly regulation of cell communication, cell population proliferation, cell differentiation, cell junction assembly, and regulation of Wnt signaling, (Fig. 2D-2, left panels and Suppl. Table 2B). KEGG pathway analysis also revealed several important cancer signaling pathways were observed to be up regulated (with significant p-values, but FDR>0.1) including PI3K-Akt, Notch, ECM-receptor interactions, regulation of actin cytoskeleton, tight junction, focal adhesion, and adherens junction signaling pathways (Fig. 2D-2, lower left panel and Suppl. Table 2B), which have been previously observed in our breast cancer studies with disruption of the CmPn network [17][19][20][21]. Down regulated DEGs mainly affected cholesterol/sterol/lipid homeostasis and transporting processes, hydrolase/transferase activity, cholesterol metabolism, regulation of blood coagulation, several cancer signaling cascades including complement and coagulation cascades, PPAR pathway, bile secretion, peroxisomes, and ABC transporters, as well as metabolic signaling pathways including fat digestion/absorption (Fig. 2D-2, right panels and Suppl. Table 2B).

Lastly, to validate diagnostic potential of key CmPn players, we analyzed DEGs comparing both primary liver tumor subtypes. After profiling DEGs between 438 HCC tumor samples with 45 CCA tumor samples, we observed down-regulation of *CCM2*, *PAQR9*, and *PGRMC1/2*, along with *AFP*, in CCA tumor tissues, while *CCM1*, *nPRs*, and *PAQRs5-8* was up regulated in CCA tissues (Fig. 2E-1, and Suppl. Table 2C). Pathway enrichment analysis revealed up regulation of mainly cellular localization, cellular component biogenesis, transmembrane transport processes, transmembrane signaling receptors, frizzled binding, G protein-coupled receptors, MAPKKK cascades, cell-cell adhesion, and proton transmembrane transport functions in CCAs (Fig. 2E-2, left panels and Suppl. Table 2C). KEGG pathway analysis of CCA tumor tissues revealed several up regulated pathways (with significant p-values, but FDR>0.1) important in cancer signaling including Wnt, AMPK, PI3K-Akt, breast cancer, prostate cancer, ECM-receptor interactions, and proteoglycans in cancer signaling pathways (Fig. 2E-2, left bottom panel and Suppl. Table 2C). Down-regulated DEGs significantly impacted exocytosis, sterol homeostasis, lipid/sterol transport, cholesterol/sterol metabolism, hemostasis, blood coagulation, wound healing processes, lipase/endopeptidase activity, regulation of proteolysis, sterol binding/transport and lipid localization (Fig. 2E-2, right panels and Suppl. Table 2C). Similar to the analysis performed with CCA tumors and normal healthy tissue, down regulated signaling pathways in CCAs when compared to HCCs also included cholesterol metabolism, several cancer signaling cascades including chemical carcinogenesis, complement and coagulation cascades, PPAR pathway, bile secretion/synthesis, peroxisomes, and ABC transporters, as well as metabolic signaling



pathways including fat digestion/absorption (Fig. 2E-2, right bottom panel and Suppl. Table 2C).

Differential expression patterns of key CmPn genes across various histological categories of hepatic cancers and their associated prognostic effects.

Utilizing TCGA databases, we compiled expression profiles for hepatic cancers by filtering histological types comparing HCC, the three major types of CCA (Intrahepatic, distal, and hilar/perihilar) and cHCC-CCAs. After comparing all subtypes of CCA to HCC and cHCC-CCA clinical tumors, we observed significant differential expression of almost all CmPn members, as well as *AFP*, except for *CCM3* (Fig. 3A). Recently, there has been mounting evidence that tumor cells can exhibit stem cell-like properties, allowing tumor cells the capacity of self-renewal and is responsible for the long-term maintenance of tumors including therapeutic resistance, tumor dormancy, and metastasis [43][44][45]. Given the importance of this, we next assessed stemness scores between HCCs, the three subtypes of CCAs, and cHCC-CCAs. Utilizing clinical RNAseq data, we obtained significant differences between stemness scores among the three major subtypes of CCAs, HCCs, and cHCC-CCA clinical tumors (Fig. 3B), suggesting that all subtypes analyzed have significant differences in terms of self-renewal and repopulation capacities.

Differential expression patterns of key CmPn genes across immune subtype classifications among hepatic cancers and their associated prognostic effects.

Given the importance of tumor microenvironments for both tumorigenesis and immunogenicity [46], we analyzed differential expression of CmPn network genes, along with *AFP*, among immune subtypes for both HCCs and CCAs. Clinical data available for HCC and CCA tumors included immune subtype classifications for wound healing (C1), IFN-gamma dominant (C2), inflammatory (C3), and lymphocyte depleted (C4) subtypes. Overall expression profiling of 355 HCC tumors, across all immune subtypes, revealed significant differential expression patterns for almost all CmPn players, along with *AFP*, excluding *CCM2* and *PAQR7/9* (Fig. 3C-1). When we repeated our analysis for overall expression profiling of 35 CCA tumors, across all immune subtypes, we only obtained significant differential expression patterns for *PAQR6*, which may be attributable to a small sample size of available clinical data for CCAs, in TCGA, regarding immune subtype classifications (Fig. 3C-2). These results illustrate not only the involvement of the CmPn network in the tumor microenvironment, but also demonstrates the potential use of expression profiling among multiple members of the CmPn signaling network for HCCs, and *PAQR6* for CCAs, as potential prognostic biomarkers across various immune subtypes in hepatic cancers.

#### Differential expression patterns of mPR and CCM genes between wound healing (C1) and IFN- $\gamma$ dominant (C2) immune subtype classifications

To further investigate significance of immune subtypes among HCCs and CCAs, we performed pairwise comparisons between various immune subtypes, given their importance in influencing the tumor microenvironment. First we compared wound healing (C1), characterized with elevated expression of angiogenic genes, a high proliferation rate, and a Th2 cell

bias to the adaptive immune infiltrate with IFN- $\gamma$  dominant (C2) immune subtype, which has the highest M1/M2 macrophage polarization, a strong CD8 signal, high proliferation rate, and high T-cell receptor diversity [47]. Analysis of HCCs demonstrated mainly up regulation in C1 subtype for members of the CSC and mPRs (thereby down regulated in C2 subtype), with significance obtained for *CCM1* and *PAQR6* (Suppl. Fig. 1A, left upper panel and Suppl. Table 4A). Interestingly, pathways functional enrichment analysis (Suppl. Table 4A) revealed mainly down regulated pathways for C1 subtype (thereby up regulated in C2 subtype) including chemokine receptor binding, MHC protein binding, and antigen processing and presentation (Suppl. Fig. 1A, lower left panel and right panels).

Interestingly, when we repeated this analysis for CCAs, we did not observe any significant differential expression for key CmPn members (Suppl. Fig. 2A and Suppl. Table 5A), most likely due to a limited sample size.

#### Differential expression patterns of mPR and CCM genes between wound healing (C1) and inflammatory (C3) immune subtype classifications

We next compared C1 subtype with the inflammatory immune subtype (C3), characterized by elevated Th1/Th17 genes, low to moderate tumor cell proliferation, lower levels of aneuploidy and lower overall somatic copy number alterations than the other immune subtypes [47]. Analysis of HCCs demonstrated significant up regulation in C1 subtype (thereby down regulated in C3 subtype) for *CCM3* and *PAQR5/6*, along with *AFP* (Suppl. Fig. 1B, left upper panel and Suppl. Table 4B). We only observed significant down regulation (thereby up regulated in C3 subtype) for *PGRMC1/2* (Suppl. Fig. 1B, left upper panel and Suppl. Table 4B). Interestingly, pathways functional enrichment analysis (Suppl. Table 4B) revealed mainly up regulation for C1 subtype (thereby down regulated in C3 subtype) for p53 signaling, Fanconi anemia pathway, and progesterone-mediated oocyte maturation (Suppl. Fig. 1B, lower left and right panels). Down regulated pathways for C1 subtype (thereby up regulated in C3 subtype) mainly included fatty acid metabolic processes, oxidoreductase activity, and complement/coagulation cascades (Suppl. Fig. 1B, lower left and right panels).

When we repeated this analysis for CCAs, with a much smaller sample size, we observed significant down regulation in C1 subtype (thereby up regulated in C3 subtype) for *PAQR6* (Suppl. Fig. 2B, left upper panel and Suppl. Table 5B), which interestingly was up regulated for HCCs in the same comparison (Suppl. Fig. 1B, left upper panel). Pathways functional enrichment analysis (Suppl. Table 5B) in CCAs revealed significant up regulated pathways in C1 subtype (thereby down regulated in C3 subtype) for PPAR, IL-17, and HIF-1 signaling pathways (Suppl. Fig. 2B, lower left panel and right panels). Interestingly, there were very few significant down regulated pathways observed in CCAs which included oxidoreductase activity, bile secretion and Rap1 signaling pathways (Suppl. Fig. 2B, lower left panel and right panels).

#### Differential expression patterns of mPR and CCM genes between wound healing (C1) and lymphocyte depleted (C4) immune subtype classifications

We then compared C1 subtype with lymphocyte depleted immune subtype (C4), characterized by a more prominent

macrophage signature, with Th1 suppressed and a high M2 macrophage response <sup>[47]</sup>. Analysis of HCCs demonstrated significant up regulation in C1 subtype (thereby down regulated in C4 subtype), for *PAQR5/6/8*, along with *AFP* (Suppl. Fig. 1C, left upper panel and Suppl. Table 4C). Significant down regulation in C1 subtype (thereby up regulated in C4 subtype) was observed for *PGRMC1/2*, *PAQR9* and *CCM2* (Suppl. Fig. 1C, left upper panel and Suppl. Table 4C). Interestingly, pathways functional enrichment analysis (Suppl. Table 4C) revealed mainly up regulation for C1 subtype (thereby down regulated in C4 subtype) for Wnt signaling, ECM proteoglycans in cancer pathways, and Hippo signaling pathways (Suppl. Fig. 1C, lower left and right panels). Down regulated pathways in C1 subtype (thereby up regulated in C4 subtype) were more prominent including fatty acid/acyl-CoA metabolic and ligase processes, cholesterol metabolism, and PPAR signaling pathways (Suppl. Fig. 1C, lower left panel and right panels).

When we repeated this analysis for CCAs, with a much smaller sample size, we observed significant down regulation in C1 subtype (thereby up regulated in C4 subtype) for *PAQR6* (Suppl. Fig. 2C, left upper panel and Suppl. Table 5C), which was up regulated for HCCs in the same comparison (Suppl. Fig. 1C, left upper panel). Pathways functional enrichment analysis (Suppl. Table 5C) in CCAs revealed significant up regulated pathways in C1 subtype (thereby down regulated in C4 subtype) for cytokine activity, TNF, IL-17, and PIK3-Akt signaling pathways (Suppl. Fig. 2C, lower left panel and right panels). Significantly down regulated pathways in C1 subtype (thereby up regulated in C4 subtype) included mitochondrial translational pathways, non-alcoholic fatty liver disease signaling, and shared signaling cascades with other diseases including Parkinson's/Alzheimer's/Huntington's disease (Suppl. Fig. 2C, lower left panel and right panels).

#### Differential expression patterns of mPR, nPRs, and CCM genes between IFN- $\gamma$ dominant (C2) and inflammatory (C3) immune subtype classifications

We next compared C2 with C3 immune subtypes. Analysis of HCCs demonstrated significant up regulation in C2 subtype (thereby down regulated in C3 subtype), for *CCM3* and *PAQR6/8*, along with *AFP* (Suppl. Fig. 1D, left upper panel and Suppl. Table 4D). Significant down regulation in C2 subtype (thereby up regulated in C3 subtype) was observed for *PGRMC1/2*, *nPRs* and *CCM1* (Suppl. Fig. 1D, left upper panel and Suppl. Table 4D). Pathways functional enrichment analysis (Suppl. Table 4D) revealed significant up regulation for C2 subtype (thereby down regulated in C3 subtype) for cell cycle phase transition, chemokine activity, and TH1/TH2 cell differentiation pathways (Suppl. Fig. 1D, lower left panel and right panels). Down regulated pathways in C2 subtype (thereby up regulated in C3 subtype) included Notch signaling, breast/endometrial/prostate cancer signaling pathways, mTOR and FoxO signaling pathways (Suppl. Fig. 1D, lower left panel and right panels).

When we repeated this analysis for CCAs, with a much smaller sample size, we observed significant down regulation in C2 subtype (thereby up regulated in C3 subtype) for *PAQR5/6*, and *PGRMC1*, while observing significant up regulation of *CCM2* (Suppl. Fig. 2D, left upper panel and Suppl. Table 5D). Pathways functional enrichment analysis (Suppl. Table 5D) in CCAs revealed significant up regulated pathways in C2 subtype (thereby down regulated in C3 subtype) for T-cell proliferation/activation, cytokine/chemokine signaling, and lymphocyte differentiation pathways (Suppl. Fig. 2D, lower left panel and right panels). Significantly down regulated pathways in C2 subtype (thereby up regulated in C3 subtype)

included cell adhesion molecules (CAMs), PI3K-Akt signaling, and carbohydrate digestion/absorption pathways (Suppl. Fig. 2D, lower left panel and right panels).

#### Differential expression patterns of mPR and CCM genes between IFN- $\gamma$ dominant (C2) and lymphocyte depleted (C4) immune subtype classifications

We next compared C2 with C4 immune subtypes. Analysis of HCCs demonstrated significant up regulation in C2 subtype (thereby down regulated in C4 subtype), for *PAQR5/8* along with *AFP* (Suppl. Fig. 1E, left upper panel and Suppl. Table 4E). Significant down regulation in C2 subtype (thereby up regulated in C4 subtype) was observed for *PGRMC1/2* and *CCM1* (Suppl. Fig. 1E, left upper panel and Suppl. Table 4E). Pathways functional enrichment analysis (Suppl. Table 4E) revealed significant up regulation for C2 subtype (thereby down regulated in C4 subtype) for neutrophil activation/degranulation, hemostasis, and CAMs pathways (Suppl. Fig. 1E, lower left panel and right panels). Down regulated pathways in C2 subtype (thereby up regulated in C4 subtype) included fatty acid processes, bile acid biosynthesis, and PPAR signaling (Suppl. Fig. 1E, lower left panel and right panels).

When we repeated this analysis for CCAs, with a much smaller sample size, we observed significant down regulation in C2 subtype (therefore up regulated in C4 subtype) for *PAQR6* and *PGRMC1* (Suppl. Fig. 2E, left upper panel and Suppl. Table 5E). Pathways functional enrichment analysis (Suppl. Table 5E) in CCAs revealed significant up regulated pathways in C2 subtype (thereby down regulated in C4 subtype) for B-cell activation and lymphocyte differentiation pathways (Suppl. Fig. 2E, lower left panel and right panels). Significantly down regulated pathways in C2 subtype (thereby up regulated in C4 subtype) included VEGFR binding/functions, cadherin binding, and SIRT1 negative regulation of rRNA pathways (Suppl. Fig. 2E, lower left panel and right panels).

#### Differential expression patterns of mPR, nPRs, and CCM genes between inflammatory (C3) and lymphocyte depleted (C4) immune subtype classifications

Finally, we performed pair-wise comparisons between C3 and C4 immune subtypes. Analysis of HCCs demonstrated significant up regulation in C3 subtype (thereby down regulated in C4 subtype) for *PAQR5/8* along with *nPRs* (Suppl. Fig. 1F, left upper panel and Suppl. Table 4F). Significant down regulation in C3 subtype (thereby up regulated in C4 subtype) was observed for *CCM2/3* (Suppl. Fig. 1F, left upper panel and Suppl. Table 4F). Pathways functional enrichment analysis (Suppl. Table 4F) revealed significant up regulation for C3 subtype (thereby down regulated in C4 subtype) for angiogenesis, ERK1/2 and MAPK cascades, as well as PI3K-Akt signaling (Suppl. Fig. 1F, lower left panel and right panels). Down regulated pathways in C3 subtype (thereby up regulated in C4 subtype) included G2/M phase transition, Fanconi anemia pathway, and ubiquitin protein activities (Suppl. Fig. 1F, lower left panel and right panels).

When we repeated this analysis for CCAs, with a much smaller sample size, we only observed significant down regulation in C3 subtype (thereby up regulated in C4 subtype) for *CCM3* (Suppl. Fig. 2F, left upper panel and Suppl. Table 5F). Pathways functional enrichment analysis (Suppl. Table 5F) in CCAs revealed significant up regulated pathways in C3 subtype (thereby down regulated in C4 subtype) for Notch signaling, blood coagulation, and semaphorin interactions

(Suppl. Fig. 2F, lower left panel and right panels). Significantly down regulated pathways in C3 subtype (thereby up regulated in C4 subtype) included non-alcoholic fatty liver disease, glycolysis/gluconeogenesis, and TCA cycle pathways (Suppl. Fig. 2F, lower left panel and right panels).

## Expression profiling of key CmPn players across HCC tumors integrating SES, follow-up and clinical survival data

We repeated expression-profiling patterns for key CmPn players, along with *AFP*, in liver tumor tissues using patient clinical data including race, family cancer history, and vascular invasion, as well as follow-up data regarding new tumor events/sites and vital status. We first evaluated expression profiles based on the four major race categories available. Interestingly, for patients diagnosed with HCCs, significant differential expression of *CCM1/2*, *PAQR7*, *PGRMC1/2*, and *nPRs*, along with *AFP*, were observed across the four major races (Fig. 3D). Interestingly, when we repeated our analysis for CCAs, we observed no significance among CmPn genes (Suppl. Fig. 3A). Next, we performed expression profiling based on family cancer history, and observed significant differential expression patterns for *PAQR6*, *PGRMC1/2*, and *nPRs* among HCC patients (Fig 3E). We then explored expression patterns among genders and observed significant differential expression for *PAQR6* and *nPRs* in HCC patients (Suppl. Fig 4D-1), with no significance for CmPn genes, except *AFP*, in CCA patients (Suppl. Fig 4D-2). We next evaluated expression profiling based on vascular invasion and tumor recurrence for HCC clinical tumors. The three main categories for vascular invasion (defined as malignant cells lining the vascular cavities of endothelial cells or portal/hepatic veins)<sup>[48]</sup> included macro (gross tissue evaluation), micro (histopathological examination of tumor and surrounding hepatic tissue) or none. Significant differential expression patterns for *PAQR5* and *PGRMC1* were observed in HCC patients (Fig. 3F), demonstrating the importance of mPRs in influencing vascular invasion.

We next evaluated differential expression among HCC patients with new tumor events including locoregional recurrence (growth of cancer cells at the same site as the primary tumor), intrahepatic recurrence (recurrence of cancer in the liver after hepatic resection), extrahepatic recurrence (recurrence of the cancer in the lungs, lymph nodes, etc.), new primary tumor, or no new tumor events. Interestingly, we observed significant differential expression in HCC patients for *CCM2*, *PGRMC1*, and *nPRs*, along with *AFP*, demonstrating the importance of the CmPn network in influencing tumor recurrence (Fig. 3G). Next, we profiled expression patterns based on new tumor event site and vital status for HCC and CCA patients. Anatomical tumor recurrence between the lungs, liver, lymph nodes, bones, and brain revealed significant differential expression for *CCM1*, *PAQR9*, and *PGRMC1*, along with *AFP*, in HCC patients (Fig. 3H), while no significance was observed for CCA patients (Suppl. Fig. 4B). Interestingly, we also evaluated residual tumor classification, which evaluates tumor status, post treatment/resection, for CCA and HCC patients with either micro (R1), macro (R2) or no residual tumors (R0). We observed no significant expression differences for HCC patients (Suppl. Fig 4E-1) but observed significant expression differences in *CCM2/3*, along with *AFP*, for CCA patients (Suppl. Fig. 4E-2). Finally, for HCC patients, we observed significant differential expression for *PAQR6/7* and *nPRs* based on vital status (Fig. 3I), while observing no significant expression differences for CCA patients (Suppl. Fig. 4C). Together, these results validate the involvement of the CmPn signaling network in liver cancer tumorigenesis, and furthermore demonstrate their involvement

in tumor recurrence mechanisms. In sum, our clinical data support the future use of CmPn members' expression data, confirmed with *AFP*, as potential prognostic biomarkers for distinguishing between primary subtypes of liver cancer and evaluating tumor recurrence in patients. Given these results, we wanted to further our investigation by assessing whether altered expression of these genes was also observed at the translational level using immunofluorescent techniques.

### Protein expression profiling of key CmPn members among normal and liver cancer tissues

To further examine the involvement of the CmPn network in liver tumorigenesis, we next examined differential expression patterns of key CmPn proteins across seven liver cancer subtypes and normal liver tissues (Suppl. Fig. 4). Utilizing liver tissue microarrays (Suppl. Fig. 4 and Suppl. Table 3), we analyzed protein expression levels of CCM1/3, PAQR7/8, PGRMC1, and nPRs, along with AFP, using immunofluorescence (IF) imaging (Suppl. Table 3). We observed significantly decreased protein expression for CCM1 (Fig. 4A-1 & 4A-2), CCM3 (Fig. 4B-1 & 4B-2), PAQR8 (Fig. 4C-1 & 4C-2), PGRMC1 (Fig. 4D-1 & 4D-2) and nPRs (Suppl. Fig. 5B) in all liver cancer subtypes, compared to normal healthy liver tissues. Interestingly, PAQR7 displayed similar decreased trends among liver cancer subtypes, with the exception of HCC, which had increased expression compared to normal liver cancer tissues (Fig. 4E-1 & 4E-2). When comparing protein expression of key CmPn players within liver cancer subtypes, PAQR7, PGRMC1 and nPRs proteins were significantly differentially expressed only when comparing HCC to all other subtypes (Fig. 4D-E and Suppl. Fig. 5B), which was also observed in our preliminary TCGA analysis between HCCs and CCAs (Fig. 3A). Interestingly, for CCM1/3 and PAQR8, we observed more significant differential expression patterns within all cancer subtypes imaged, with undifferentiated pleomorphic sarcoma (UPS) displaying the lowest levels for CCM3 (Fig. 4B) and PAQR8 expression (Fig. 4C), while hepatic angiosarcoma (HAS) displayed the weakest expression of CCM1 (Fig. 4A), which was also true for PGRMC1 (Fig. 4D) and PAQR7 (Fig. 4E). These results demonstrate that PAQR7, PGRMC1 and nPRs can be used as an HCC specific biomarker, while CCM1/3 and PAQR8 can be used as a biomarker signature to differentiate undifferentiated pleomorphic sarcoma (UPS) and hepatic angiosarcoma (HAS) from other hepatic cancer subtypes.

#### Altered expression levels of CCM3 and PAQR7 in biphenotypic liver cancer subtype

Furthermore, we compared CCM3 and PAQR7 protein expression between HCC and cHCC-CCA (Fig. 5 and Suppl. Table 3), since cHCC-CCA is known to be a biphenotypic liver cancer subtype, presenting hepatocytic and biliary differentiation [49]. IF methods revealed cHCC-CCA tissues exhibited the lowest abundance of both proteins compared to NORM and HCC tissues (Fig. 5A-1 and 5A-2). While examining CCM3 and PAQR7 expression, we observed NORM tissue samples contained more co-localized "clumped" patterns for CCM3 and PAQR7 (Fig. 5B-60X imaging) compared to both HCC and cHCC-CCA tissues, suggesting down-regulation of key members of the CmPn network during liver tumorigenesis. Our data also indicate the loss of specific cellular co-localization patterns of both CCM3 and PAQR7 can be used as a pathological biomarker signature for diagnosing cHCC-CCAs and HCCs.

Copy number variations of CmPn players in HCC tissues confirms crosstalk among the CmPn signaling network during liver cancer tumorigenesis



To evaluate the relationship between key CmPn players within the CmPn signaling network, we utilized publicly available microarray data from 14 HCC patients to generate an oncogrid (Fig. 6A). Each column contains copy number variation (CNV) information, mutation type for each CmPn gene, as well as clinical information. Our analysis identified key CmPn mutations seen in HCC patients, but also solidified the existence of crosstalk interactions among key members of the CmPn network during liver tumorigenesis. We observed not only missense (GOF or LOF), frameshift and nonsense (LOF) CmPn gene mutations, but also observed compound mutations (one GOF and one LOF) co-exist among CmPn genes (Fig. 6A). Both *CCM1* and *PAQR6* were the two members impacted with gain of function (GOF) alterations, while *nPRs* was the only member frequently impacted with a loss of function (LOF) alteration (Fig. 6A). Next we wanted to assess clinical impacts of altered expression levels of key CmPn members.

### Differential expression patterns of *mPR* and *CCM* genes across clinical tumors and their associated survival effects

Utilizing publicly available microarray data from HCC patients (not enough data available for CCAs), we incorporated gene expression and clinical data simultaneously to generate Kaplan-Meier (KM) survival curves to understand the impact of the CmPn network on overall survival (OS) in liver cancer patients (Fig. 6B). In each panel, the left KM curve was generated using kmplotter [36], while the right KM curve in each panel was generated using TCGA data to validate our kmplotter analysis. Our analysis revealed that increased expression of *CCM3* (Fig. 6B-1) and *PAQR5/7/9* (Fig. 6B-3), while decreased expression of *PGRMC1/2* (Fig. 6B-2) demonstrated significantly worse prognostic outcomes in HCC patients. Of these six key CmPn players, we were able to validate all survival curves, except *PAQR5*, utilizing TCGA data. These results further substantiate the involvement of the CmPn signaling network in liver cancer tumorigenesis and further solidify their potential prognostic application in HCCs.

## Discussion

The importance of the CmPn signaling network in the tumorigenesis of multiple cancers has been well documented recently [16][17][20], demonstrating not only equal importance of both mPRs and nPRs in PRG signaling, but also demonstrating that organs which utilize/metabolize PRG are susceptible to changes in CmPn members' expression during tumorigenesis [12]. In our foundational studies, we defined the novel CmP signaling network (absence of nPRs) in African American Women-derived triple negative breast cancer (TNBC) cells [16], which overlapped with our observations of altered CSC and mPRs expression changes in nPRs(+) breast cancer cells [21]. Furthermore, using Caucasian American Women-derived TNBC cells, we demonstrated that all three breast cancer subtypes shared alterations to key tumorigenesis pathways including cytokine-mediated responses, Wnt, Integrin, Gonadotropin Releasing Hormone, and angiogenesis signaling pathways under mPR-specific PRG actions (PRG actions through mPRs only) [16][17][21]. In regards to liver tumorigenesis, our previous pilot study demonstrated the largest differential expression of CmPn members was observed in liver tumor tissues, proceeded by endometrial and breast tumor tissues [20]. In this study, utilizing RNAseq data and IF approaches, we were able to further solidify the importance of the CmPn signaling network during

liver cancer tumorigenesis by examining expression profiling for key CmPn members across multiple liver cancer subtypes, at both the transcriptional and translational levels. We observed that several members of the CmPn network (including CCM1/3 and PAQR7/8) were altered at both the transcriptional (Figs 2-3) and translational levels (Figs. 4-5), between both normal and tumor tissues, as well as between liver cancer subtypes, which was also demonstrated for PGRMC1 (Fig. 4D). Additionally, we also observed altered expression of nPRs (Suppl. Fig. 5B) in all liver cancer subtypes, compared to normal healthy liver tissues, as well as between HCC and all other subtypes (Suppl. Fig. 5B). Interestingly, we were able to prove specificity of AFP as a potential biomarker among CCA tissues, of which we observed decreased expression at both the transcriptional (Fig. 2D-1) and translational levels (Suppl. Fig. 5A), when compared to normal tissues, which is opposite to the well-documented trends observed in HCC.

Using systems biology approaches to compare CCAs and HCCs with normal healthy tissues, we observed similar alterations to key tumorigenesis pathways, observed in our breast cancer omics analysis [\[16\]\[17\]\[20\]\[21\]](#), including altered Wnt signaling, Progesterone-mediated oocyte maturation, oocyte meiosis, cholesterol metabolism, cell cycle phase transition, and altered regulation of cell proliferation (Figs. 2C-D). Interestingly, our analysis with liver tissues also revealed disruption of common cancer signaling pathways, when comparing CCAs and HCCs with normal healthy tissues, which are known to influence liver tumorigenesis including sterol/cholesterol homeostasis [\[50\]](#), bile secretion [\[51\]\[52\]](#), ABC transporters [\[53\]](#), PPAR signaling [\[54\]](#), and frizzled binding [\[55\]](#) (Fig. 2C-D). When we repeated our analysis, comparing CCAs with HCCs, we noticed opposite regulation between the two common liver cancer subtypes in which cholesterol/triglyceride/lipid homeostasis, cholesterol transport, PPAR signaling, bile secretion, as well as ABC transporters, were down regulated in CCAs compared to HCCs (Fig. 2E). Similarly, frizzled binding, MAPKKK signaling, and protein kinase binding were up regulated in CCAs compared to HCCs (Fig. 2E). These results solidify the importance of these common signaling cascades that are oppositely regulated among these subtypes and demonstrate the importance of evaluating altered gene expression of key CmPn members involved in these signaling cascades [\[16\]\[17\]\[19\]\[20\]\[21\]](#).

## Potential prognostic biomarkers for hepatic cancers

Biomarkers, identified through genetics/epigenetics, genomics/proteomics etc., are essential prognostic tools needed for liver cancer patients to help with not only early detection but to also predict patient response to therapy as well as future course of the disease [\[56\]\[57\]](#). As previously mentioned, 5-year survival rates for patients with liver cancer, in general, is 5-30% [\[3\]](#), more specifically, about 15% for HCC and 5-15% for CCA patients [\[4\]](#). Patients with HCC who receive treatment earlier are more likely to have longer recurrence-free periods and better overall survival rates [\[5\]](#). However, the prognosis of HCC is poor, as HCC has been referred to as a “chemoresistant” tumor [\[6\]](#).

Currently, there are limited biomarkers approved for diagnosing hepatic cancers, with AFP being the sole gold standard [\[58\]\[59\]](#), despite its known limitations due to lack of sensitivity and specificity in detecting various grades/stages of HCC, as well as limitations in accurately differentiating between primary liver cancer types [\[27\]\[60\]](#). The lack of reliable biomarkers for the diagnosis/prognosis of hepatic cancers emphasizes the urge for identification of new biomarkers for various subtypes in order to increase early diagnosis of these high-risk cancers to provide patients with better treatment

options [57]. AFP is often used in conjunction with other biomarkers in the early diagnosis of HCC because of its low specificity and sensitivity, however, AFP levels are not very high in 80% of HCC patients with small tumor sizes [27][61]. Positive serum AFP levels are only seen in about 19% of CCA cases, making it difficult to be effectively utilized as a biomarker for liver cancers other than HCC [62]. Unfortunately, rarer liver cancer subtypes have the least available options for early detection; therefore, identification of new potential biomarkers is of the utmost importance in providing early screening and better treatment and prognosis options for these liver cancer patients. Together, these limitations shed light on the importance of our observed findings of differential expression of multiple members of the CmPn network, among both HCC and CCA clinical tumor samples, at both the transcriptional and translational levels.

Utilizing RNA-seq and IF approaches, we observed that *CCM1* and *nPRs* were seen to be down regulated, at both the transcriptional and translational levels, in HCC and CCA tissues when compared to normal tissues, indicating that these CmPn members could be useful diagnostic biomarkers, but did not support its potential use as a prognostic biomarkers in differentiating between cancer subtypes. Up regulated levels of *PAQR7* in HCC tissues only, at both the transcriptional (Fig. 2C-1) and translational levels (Fig. 4E), as well as our observation that increased expression of *PAQR7* is associated with decreased survival in HCC patients (Fig. 6B-3), supports its use as a potential prognostic biomarker to differentiate between HCCs and CCAs. Previous research has associated decreased expression of *PGRMC1* with advanced stages and poorer prognosis, and therefore, has been proposed as a potential prognostic biomarker for HCC and various other cancers [25][63][64]. We observed down regulation of *PGRMC1* in HCCs at the translational level (Fig. 4D) but observed that mRNA levels of *PGRMC1* are actually slightly increased compared to normal healthy tissues (Figs. 2A-1 and 2C-1). Interestingly, we observed more dramatic down regulation of *PGRMC1*, occurring at both the transcriptional (Figs. 2A-2 and 2D-1) and translational levels (Fig. 4D), in CCAs, further suggesting its potential use as a prognostic biomarker for CCAs. These findings are extremely significant since CCAs have less available validated biomarkers for early detection [62]. Interestingly, in addition to analyzing expression levels of key CmPn members, we assessed AFP expression levels in CCA tissues, and saw synchronous down-regulation at both the transcriptional (Figs. 2A-1 and 2D-1) and translational levels (Suppl. Fig. 5A), which corroborates with previous findings that *AFP* levels are typically lower in CCAs than normal tissues, contrary to increased expression observed in HCCs [27].

## Pathways functional enrichment analysis using DEGs for HCCs and CCAs demonstrate perturbation in key tumorigenic signaling cascades

Pathways functional enrichment results obtained from significantly altered genes in HCC clinical samples demonstrated perturbation to key cancer signaling pathways including cell cycle, DNA replication, spliceosomes/lysosomes, pancreatic cancer signaling pathways, and pancreatic secretion pathways (Fig. 2C-2). Interestingly, we also observed dysregulation in hormone signaling pathways including Oocyte meiosis and PRG-mediated oocyte maturation (Fig. 2C-2), which were also altered pathways observed in all of our breast cancer studies [16][17][21]. Functional enrichment results obtained from significantly altered genes in CCA clinical samples

demonstrated perturbation to key cancer signaling pathways including cell population proliferation/differentiation, cell junction assembly, Wnt signaling, PI3K-Akt, and Notch signaling (Fig. 2D-2), which were also observed to be dysregulated in all of our breast cancer studies [16][17][21]. We also observed newly dysregulated pathways, not observed in our breast cancer studies, including disruption of ECM-receptor interactions, complement/coagulation cascades, PPAR pathways, bile secretion, peroxisomes, sterol/cholesterol homeostasis and ABC transporters (Fig. 2D-2), which are known to influence liver tumorigenesis. Interestingly, when we repeated our analysis to compare CCAs with HCCs, we noticed opposite regulation between the two common liver cancer subtypes including down regulation of Cholesterol/triglyceride/lipid homeostasis, cholesterol transport, PPAR signaling, bile secretion as well as ABC transporters in CCAs compared to HCCs (Fig. 2E-2). Similarly, frizzled binding, MAPKKK signaling, and protein kinase binding were observed to be up regulated in CCAs compared to HCCs (Fig. 2E-2), solidifying the importance of these common signaling cascades in distinguishing HCCs from CCAs.

### Pathways functional enrichment analysis using DEGs in the immune landscape of hepatic cancers

Given the importance of tumor microenvironments for both tumorigenesis and immunogenicity in hepatic cancers [46], one of our main tasks was also to assess differential expression of CmPn network genes, along with *AFP*, among immune subtypes for both HCCs and CCAs.

#### Wound healing (C1) subtype compared to IFN- $\gamma$ dominant (C2) subtype

C1 (wound healing) immune subtypes are characterized by elevated expression of angiogenic genes, while C2 (IFN- $\gamma$  dominant) subtypes have high M1/M2 macrophage polarization [47]. Cirrhosis is a form of recurrent wound healing and, when seen in liver cancer patients, can decrease life expectancy [65]. An M2 to M1 phenotypic shift occurs during HCC tumor progression, which promotes cancer cell proliferation [66]. Intriguingly, we noted during our comparisons that there were very few significant up regulated pathways in HCCs, while CCAs demonstrated significance for both up and down regulated pathways (Suppl. Figs. 1A and 2A). When comparing the two immune subtypes, we observed shared down regulated pathways in C1 subtype, for both HCCs and CCAs, including chemokine/cytokine receptor activity, MHC protein complex binding, Th1/2/17 cell differentiation, and CAMs pathways (Suppl. Figs. 1A and 2A). Interestingly, down regulation of TNF-activated receptor activity and NF-kappa B signaling were only observed in CCAs (Suppl. Fig. 2A), both of which are associated with aggressive CCA development and poor prognosis [67][68].

#### Wound healing (C1) subtype compared to inflammatory (C3) subtype

We next compared C1 immune subtype with the C3 (inflammatory) immune subtype, characterized by elevated Th1 and Th17 genes and low to moderate tumor cell proliferation [47]. High levels of Th1 and Th17 are associated with HCC invasiveness and can be indicative of increased tumor progression [69]. Interestingly, among the top altered pathways in C1 subtype, we did not observe any shared up-regulated pathways between HCCs and CCAs (Suppl. Figs. 1B and 2B). Interestingly, shared down-regulated pathways in C1 subtype, for both HCCs and CCAs, included peroxisomes, glycine/serine/threonine metabolism, and PPAR signaling pathways (Suppl. Figs. 1B and 2B), of which PPAR $\gamma$  has been

demonstrated to display inhibitory effects on HCC metastasis [70][71].

#### Wound healing (C1) subtype compared to lymphocyte depleted (C4) subtype

Similarly, we compared C1 subtype to the C4 (lymphocyte depleted) subtype, which is classified as having suppressed Th1 levels and a high M2 response [47]. An elevated M2 response in HCC has been associated with tumor resistance to sorafenib, which is currently utilized as a standard treatment for liver cancers [72]. We observed shared up regulated pathways in C1 subtype, for both HCCs and CCAs, including AGE-RAGE signaling, ECM organization, and proteoglycans in cancer (Suppl. Figs. 1C and 2C). Interestingly, previous studies have shown that AGE-RAGE signaling is upregulated in liver fibrosis, while RAGE silencing reduces liver tumor growth [73]. Shared down regulated pathways in C1 subtype, for both HCCs and CCAs, included fatty acid degradation and lipid/lipoprotein metabolism (Suppl. Figs. 1C and 2C). To our surprise, Wnt signaling was observed to be up regulated only in HCCs (Suppl. Figs. 1C), which was also a main altered pathway observed in our previous breast cancer studies investigating effects of altered CmPn expression [16][17][21].

#### IFN- $\gamma$ dominant (C2) subtype compared to inflammatory (C3) subtype

In our analysis, we observed several shared up regulated pathways in C2 subtype, for both HCCs and CCAs, including cytokine-mediated signaling, CAMs, and Th17 cell differentiation pathways (Suppl. Figs. 1D and 2D). Previous studies have demonstrated that the production of IL-17 by Th17 cells induces liver inflammation by stimulating multiple types of liver nonparenchymal cells that secrete cytokines and chemokines [74]. Interestingly, shared down regulated pathways in C2 subtype, for both HCCs and CCAs, included Rap1 signaling, cadherin binding, and adherens junction pathways (Suppl. Figs. 1D and 2D). Previous studies have shown that Rap1 suppresses tumorigenesis and is up regulated in HBV-related HCC pathogenesis [75]. Intriguingly, down regulation of FoxO signaling pathways in C2 subtype was only observed for HCCs (Suppl. Figs. 1D), which have been associated with poor prognosis in liver cancer patients, as they develop resistance to chemotherapy treatment [76].

#### IFN- $\gamma$ dominant (C2) subtype compared to lymphocyte depleted (C4) subtype

Based on our analysis, we observed shared up regulated pathways in C2 subtype, for both HCCs and CCAs, including cytokine/chemokine receptor activity, CCR chemokine receptor binding, and hematopoietic cell lineage pathways (Suppl. Figs. 1E and 2E). Interestingly, recent studies have shown that overexpression of certain CCRs in HCC tissues correlates with vascular invasion [77]. Shared down regulated pathways in C2 subtype, for both HCCs and CCAs, included fatty acid degradation, valine/leucine/isoleucine degradation, and pyruvate metabolism (Suppl. Figs. 1E and 2E). Interestingly, down regulation of SIRT1 negative regulation of rRNA expression was only observed for C2 subtype in HCCs (Suppl. Figs. 1E), which has been found to be an independent prognostic factor for HCCs, as SIRT1 promotes liver tumor cell survival [78].

#### Inflammatory (C3) subtype compared to lymphocyte depleted (C4) subtype

For our final comparison, we observed shared up regulated pathways in C3 subtype, for both HCCs and CCAs, including integrin binding, general pathways in cancer, as well as AGE-RAGE signaling pathways (Suppl. Figs. 1F and 2F). We also observed shared down regulated pathways in C3 subtype, for both HCCs and CCAs, including proteasomes, oxidative phosphorylation, and translation initiation factor activity pathways (Suppl. Figs. 1F and 2F). Finally, similar to our previous study analyzing altered CmP expression levels and resulting effects on signaling pathways in African American Women-derived TNBCs [16], we again observed altered PI3K-Akt, MAPK, and angiogenesis pathways for C3 subtype, but only in HCCs (Suppl. Figs. 1F).

## Further validation of the CmPn signaling network in liver cancer

As previously mentioned, differential expression of key CmPn members has been shown to be involved in the progression of multiple types of cancers, as well as leading to worse prognostic outcomes [22][23][25][63]. The role of mPRs in tumorigenesis has been increasingly studied, more recently in liver cancer given that the liver is a primary target and metabolic organ of PRG [12]. Previous research has demonstrated that *PAQR8* and *PAQR9* are independent prognostic factors for HCC [79], which was supported in our RNA-seq analysis, as well as our generated KM survival curves for *PAQR9*, which demonstrated increased expression resulted in decreased overall survival (Fig. 6B-3). Finally, CNV analysis of HCC patients with confirmed CmPn genomic (either GOF or LOF) mutations could lead to perturbation of the CmPn signaling network in hepatic cancers. These results solidify the existence of crosstalk interactions among key members of the CmPn network during liver cancer tumorigenesis.

## Conclusion

In our previous studies, we defined the novel CmP(n) signaling network in breast cancers [16][17][21], which overlapped with our foundational study on the CSCs involvement in tumorigenesis signaling cascades [20], and observed that differential expression patterns of key CmPn members could be used as potential prognostic biomarkers for several cancers [16][17][21]. In this study, we were able to demonstrate alterations to key CmPn members as well as disruption of key tumorigenic signaling pathways between HCCs and CCAs, along with CmPn alterations among various immunological subtypes of hepatic cancers. We were also able to demonstrate that there were significant differential expression patterns for key CmPn members between HCCs and CCAs, at both the transcriptional and translational levels, as well as between rarer hepatic cancer subtypes. These results provide a new set of potential prognostic biomarkers for multiple subtypes of hepatic cancers, which may revolutionize molecular mechanisms and currently known concepts of liver cancer tumorigenesis and treatment options, leading to hopeful new therapeutic strategies.

## Data availability

Readers can access the data supporting the conclusions of the study through supplemental materials and some omics



data are in process to deposit into NIH genomic or proteomic databases repertoire, and can be acquired by contact the corresponding author.

#### Conflicts of interest

The author(s) declare(s) that there is no conflict of interest regarding the publication of this article.

#### Funding Statement

This work was supported by NIH (1R21NS061191) and the Coldwell foundation (JZ).

#### Acknowledgments

We wish to thank Chantal Alvarado, Hira Ali, Mike Yao, Yanchun Qu, Shen Sheng, Ahmed Badr, Junli Zhang, Amna Siddiqui, Pallavi Dubey, Saafan Malik, and Edna Lopez at Texas Tech University Health Science Center El Paso (TTUHSCPE) for their technical help during the experiments. The results shown here are in part based upon data generated by the TCGA Research Network: <https://www.cancer.gov/tcga>

#### Author contributions

JZ: Conceptualization, Methodology, Investigation, Writing- reviewing and editing; JAF: Investigation, visualization, bioinformatics software analysis, data curation, validation, writing- original draft preparation, reviewing and editing, VR: Visualization, software, data curation, validation, Writing- original draft preparation, reviewing and editing; AL: Bioinformatics software analysis, data curation.

## Figure legends

**Figure 1: Clinical profiling for hepatic cancers utilizing NCI-GDC data.** Utilizing the Genomic Data Commons (GDC) data portal from the National Cancer Institute (NCI), we assessed all available clinical data for patients diagnosed with hepatic cancers without any gene filters (general clinical observations, panels A1-4 & B1-5) or filtered to only analyze patient samples with differential expression for any of the key CmPn players (CmPn network associated clinical observations, panels A5-8 & B6-9). **A-1)** Survival data (n=420, left panel) and age at diagnosis (n=829, right panel). **A-2)** Year of diagnosis (automatically divided into 5 categories; n=423). **A-3)** Tissue of origin (n=1606). **A-4)** Year of death (automatically divided into 5 categories; n=106). **A-5)** Survival data (n=219, left panel) and age at diagnosis (n=220, right panel). **A-6)** Year of diagnosis (n=221). **A-7)** Tissue of origin analysis (n=223). **A-8)** Year of death analysis (n=60 total). *Socioeconomic status (SES) and diagnostic profiling for hepatic cancers utilizing clinical data from the NCI-GDC data portal.* **B-1)** Ethnic (n=1022, left panel) and primary racial categories (n=1047, right panel). **B-2)** Gender analysis (n=1606). **B-3)** Prior malignancy status (for available data; n= 419). **B-4)** Primary diagnosis (liver vs. intrahepatic bile

ducts; n= 1582). **B-5)** Tumor classification (n= 397 total). **B-6)** Ethnic (n=214, left panel) and primary racial category analysis (n=217, right panel). **B-7)** Gender analysis (n=223). **B-8)** Prior malignancy analysis (n=223). **B-9)** Primary diagnosis analysis (n=223 total). For all graphs, the comparisons were performed automatically by the NCI-GDC software, for all publicly available clinical data, utilizing 14 projects (for general clinical observations) or 2 projects (for CmPn network associated clinical observations) for patients diagnosed with hepatic cancers (See suppl. Table 1). **Abbreviations:** cholangiocellular carcinoma (CCA), hepatocellular carcinoma (HCC), combined HCC+CCA (cHCC-CCA), clear cell carcinoma (CCC).

**Figure 2. RNAseq expression profiling for key CmPn players utilizing multiple TCGA databases for hepatic cancers.** We performed expression profiling for key *CSC*, *mPR* and *nPR* players, along with established liver cancer biomarker *AFP*, using two types of 'normal' tissues utilizing the TCGA-TARGET-GTEX and TCGA-PANCAN databases; solid tissue "normal" which are taken from "normal" tissue, near the tumor site, and normal healthy tissue from individuals without cancer. **A1-2)** Differentially expressed genes (DEGs) of key CmPn players (along with *AFP*) for HCCs (n=531) and CCAs (n=205), based on tissue type using the TCGA-TARGET-GTEX database. **B1-2)** Differential expression of key CmPn players (along with *AFP*) for HCCs (n=412) and CCAs (n=45), based on tissue type using the TCGA-PANCAN database. **C-1)** Significant DEGs were profiled in a pair-wise fashion between normal healthy liver tissue (n=110) and HCC primary tumors (n=369). **C-2)** Pathway functional enrichment comparisons were performed using Enrichr assessing biological processes (upper panel) molecular functions (middle panel), and KEGG signaling pathways (lower panel). **D-1)** Significant DEGs were profiled in a pair-wise fashion between normal healthy liver tissue (n=110) and CCA primary tumors (n=36). **D-2)** Pathway functional enrichment comparisons were performed assessing biological processes (upper panel) molecular functions (middle panel), and KEGG signaling pathways (lower panel). **E-1)** Significant DEGs were profiled from a separate TCGA database, with increased sample sizes, between HCC (n=438) and CCA (n=45) tumors **E-2)** Pathway functional enrichment comparisons were compiled using biological processes (upper panel) molecular functions (middle panel), and KEGG signaling pathway data (lower panel). For all boxplots, X-axis details genes profiled, while Y-axis details normalized RNAseq expression data; significantly altered genes were identified using One-way ANOVA analysis and are color-coded red. For Panels C-E, red colored genes were up-regulated while blue colored genes are down regulated; for pathways analysis, the top 15 pathways are provided and color-coded (red-up/blue-down) and pathways with p-values <0.05 and FDR<0.1 are bolded. All graphs were produced using the Xena platform.

**Figure 3. RNAseq expression profiling for key CmPn players for HCC and CCA histological/immunological subtypes integrating SES and follow-up clinical data.** We investigated key *CSC*, *mPR* and *nPR* players expression analysis, along with *AFP*, between histological/immunological subtypes (panels A, C1 and C2) and assessed stemness scores (panel B) between major HCC and CCA subtypes. Additionally, we performed expression profiling using demographic and follow-up data to assess differential expression across major races and evaluate impact on tumor recurrence (panels D-I). **A)** Significant DEG profiling between the three major subtypes of CCA compared to HCC and cHCC-CCA clinical tumors (n=465). **B)** Stem cell-associated molecular scores between the three major subtypes of CCAs compared to HCC and cHCC-CCA clinical tumors (n=462). **C-1)** Significant DEG profiling between standard immune subtype classifications for HCCs (n=355). **C-2)** Significant DEG profiling between standard immune subtypes for CCAs

(n=35). **D)** Significant DEG profiling among the 4 most prevalent races diagnosed with HCC (n=399). **E)** Significant DEG profiling for patients diagnosed with HCC and with documented family cancer history (n=358). **F)** Significant DEG profiling for HCC patients presenting with either micro/macro or no vascular invasion (n=412). **G)** Significant DEG profiling for HCC patients presenting with a new primary tumor, locoregional [cancer cells at the same site as the original (primary) tumor], intrahepatic (recurrence in liver after hepatic resection), or extrahepatic recurrence (most commonly in the lungs, lymph nodes and bones, n=174). **H)** Significant DEG profiling for HCC patients among the 5 major locations for tumor recurrence (n=150). **I)** Significant DEG profiling for HCC patients based on vital status (n=412). For all boxplots (panels A, C, and D-I), X-axis details genes profiled, while Y-axis details Log2 normalized RNAseq expression data. For Panel B, X-axis details HCC and CCA subtypes profiled, while Y-axis details RNAss-stemness signature scores (RNA based). All graphs were produced using the Xena platform.

**Figure 4. Significantly altered CmPn protein expression among hepatic cancer subtypes.** Comparative CmPn protein expression patterns were measured with immunofluorescence-labeled antibodies, normalized against nuclear staining (DAPI), and quantified using Nikon Elements Analysis software. **A-1)** Representative IF images of CCM1 protein expression among different liver cancer types and NORM tissues. **A-2)** Normalized quantification for all images. **B-1)** Representative IF images of CCM3 protein expression among different liver cancer types and NORM tissues. **B-2)** Normalized quantification for all images. **C-1)** Representative IF images of PAQR8 protein expression among different liver cancer types and NORM tissues. **C-2)** Normalized quantification for all images. **D-1)** Representative IF images of PGRMC1 protein expression among different liver cancer types and NORM tissues. **D-2)** Normalized quantification for all images. **E-1)** Representative IF images of PAQR7 protein expression among different liver cancer types and NORM tissues. **E-2)** Normalized quantification for all images. For all graphs, asterisks above each cancer type indicate significance of differential expression between specific cancer type and NORM tissues. Cancer subtype comparisons chart above each graph summarizes the relationships within cancer subtypes. Sample sizes provided beneath liver tissue type. In all bar plots and charts, \*\*\*, \*\*, \* indicates  $P \leq 0.001$ ,  $\leq 0.01$  and  $\leq 0.05$ , respectively, using one-way ANOVA.

**Figure 5. Relative abundance of CCM3 and PAQR7 between HCC and cHCC-CCA subtype.** CCM3 and PAQR7 protein expression patterns were measured with immunofluorescence-labeled antibodies, normalized against nuclear staining (DAPI), and quantified using Nikon Elements Analysis software. IF imaging was conducted at 20x (left panels) and 60x magnification (right panels) on NORM, HCC and cHCC-CCA tissues. **A-1)** Representative IF images of CCM3 and PAQR7 protein expression for NORM, HCC and cHCC-CCA tissues captured at 20x magnification. **A-2)** Normalized quantification of CCM3 and PAQR7 for NORM, HCC and cHCC-CCA tissues. **B)** Representative 60x IF imaging of areas from panel A to further examine clustering patterns of key CmPn proteins in liver tissues. Sample sizes provided above bar graphs. In all bar plots, \*\*\*, \*\*, \* indicates  $P \leq 0.001$ ,  $\leq 0.01$ , and  $\leq 0.05$ , respectively, using two-way ANOVA.

**Figure 6. Oncogrid with copy number variations (CNV) and prognostic effects for key CmPn players utilizing microarray data for HCC patients.** Utilizing the Genomic Data Commons (GDC) data portal from the National Cancer Institute (NCI), we assessed all available clinical and CNV data for patients diagnosed with hepatic cancers with differential expression for any of the key CmPn players. Additionally, publicly available microarray data from HCC patients was analyzed using either KMplotter or Xena browser (TCGA database) to integrate gene expression and clinical data

simultaneously to generate Kaplan-Meier (KM) survival curves. **A)** CNV analysis for HCC patients with CmPn mutations. The clinical data legend is provided beneath the oncogrid as well as coloring scheme for mutation outcomes and CNV impacts. **B-1)** Prognostic effects for *CCM3* in both KMplotter (left panel) and TCGA databases (right panel). **B-2)** Prognostic effects for *PGRMC1/2* in both KMplotter (left panels) and TCGA databases (right panels). **B-3)** Prognostic effects for *PAQR5/7/9* in KMplotter (left panels) and TCGA databases (right panels). For panels B1-3, Logrank P-values were automatically calculated and displayed for KMplotter analysis as well as hazard ratios (and 95% confidence intervals). Alternatively, P-values and logrank test statistics were automatically calculated and displayed for TCGA analysis. Red line demonstrates high gene expression (all panels), while black line (TCGA analysis) or blue line (KMplotter analysis) demonstrates low gene expression.

---

Received: November 13, 2022

## References

- <sup>a</sup> Sung, H., et al., *Global Cancer Statistics 2020: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries*. *CA Cancer J Clin*, 2021. 71(3): p. 209-249.
- <sup>a</sup> Petrick, J.L. and K.A. McGlynn, *The changing epidemiology of primary liver cancer*. *Curr Epidemiol Rep*, 2019. 6(2): p. 104-111.
- <sup>a, b</sup> Mattiuzzi, C. and G. Lippi, *Current Cancer Epidemiology*. *J Epidemiol Glob Health*, 2019. 9(4): p. 217-222.
- <sup>a, b</sup> Mavros, M.N., et al., *Treatment and Prognosis for Patients With Intrahepatic Cholangiocarcinoma: Systematic Review and Meta-analysis*. *JAMA Surg*, 2014. 149(6): p. 565-74.
- <sup>a, b</sup> Numbere, N., D. Zhang, and D. Agostini-Vulaj, *A rare histologic subtype of hepatocellular carcinoma, sarcomatoid hepatocellular carcinoma: report of a case*. *Hepat Oncol*, 2020. 8(2): p. HEP33.
- <sup>a, b</sup> Cabral, L.K.D., C. Tiribelli, and C.H.C. Sukowati, *Sorafenib Resistance in Hepatocellular Carcinoma: The Relevance of Genetic Heterogeneity*. *Cancers (Basel)*, 2020. 12(6).
- <sup>a</sup> Qiang, L., et al., *Factors associated with disease survival after surgical resection in Chinese patients with hepatocellular carcinoma*. *World J Surg*, 2006. 30(3): p. 439-45.
- <sup>a</sup> McGlynn, K.A., J.L. Petrick, and H.B. El-Serag, *Epidemiology of Hepatocellular Carcinoma*. *Hepatology*, 2021. 73 Suppl 1: p. 4-13.
- <sup>a</sup> Vogel, A. and A. Saborowski, *Cholangiocellular Carcinoma*. *Digestion*, 2017. 95(3): p. 181-185.
- <sup>a</sup> Rujeerapaiboon, N. and P. Wetwittayakhleng, *Primary Hepatic Angiosarcoma: A Rare Liver Malignancy - Varying Manifestations but Grave Prognosis*. *Case Rep Gastroenterol*, 2020. 14(1): p. 137-149.
- <sup>a, b</sup> Li, Z., et al., *Clinicopathological features and surgical outcomes of four rare subtypes of primary liver carcinoma*. *Chin J Cancer Res*, 2018. 30(3): p. 364-372.
- <sup>a, b, c</sup> Yeh, Y.T., et al., *Progesterone and related compounds in hepatocellular carcinoma: basic and clinical aspects*.

*Biomed Res Int*, 2013. 2013: p. 290575.

13. <sup>a</sup>Guo, Y., et al., *Anti-Hepatocellular Carcinoma Effect and Molecular Mechanism of the Estrogen Signaling Pathway*. *Front Oncol*, 2021. 11: p. 763539.
14. <sup>a</sup>Paydas, S., et al., *Estrogen and Progesterone Receptor Contents in Hepatocellular Carcinoma*. *Med J Islamic World Acad Sci*, 1992. 5(4): p. 300-304.
15. <sup>a</sup>Boix, L., et al., *Sex hormone receptors in hepatocellular carcinoma*. *Journal of Hepatology*, 1993. 17(2): p. 187-191.
16. <sup>a, b, c, d, e, f, g, h, i, j, k, l, m, n, o, p</sup>Abou-Fadel, J., et al., *CmP signaling network unveils novel biomarkers for triple negative breast cancer in African American women*. *Cancer Biomark*, 2022.
17. <sup>a, b, c, d, e, f, g, h, i, j, k, l</sup>Abou-Fadel, J., et al., *CmP Signaling Network Leads to Identification of Prognostic Biomarkers for Triple-Negative Breast Cancer in Caucasian Women*. *Genet Test Mol Biomarkers*, 2022. 26(4): p. 198-219.
18. <sup>a</sup>Padarti, A. and J. Zhang, *Recent advances in cerebral cavernous malformation research*. *Vessel Plus*, 2018. 2.
19. <sup>a, b, c</sup>Abou-Fadel, J., et al., *Systems-wide analysis unravels the new roles of CCM signal complex (CSC)*. *Heliyon*, 2019. 5(12): p. e02899.
20. <sup>a, b, c, d, e, f, g, h, i</sup>Abou-Fadel, J., et al., *Emerging roles of CCM genes during tumorigenesis with potential application as novel biomarkers across major types of cancers*. *Oncol Rep*, 2020. 43(6): p. 1945-1963.
21. <sup>a, b, c, d, e, f, g, h, i, j, k, l</sup>Abou-Fadel, J., et al., *CCM signaling complex (CSC) coupling both classic and non-classic progesterone receptor signaling*. *bioRxiv*, 2020: p. 2020.05.24.112847.
22. <sup>a, b</sup>Del Moral-Morales, A., et al., *The Role of mPRdelta and mPRepsilon in Human Glioblastoma Cells: Expression, Hormonal Regulation, and Possible Clinical Outcome*. *Horm Cancer*, 2020. 11(2): p. 117-127.
23. <sup>a, b</sup>Sinreih, M., et al., *Membrane progesterone receptors beta and gamma have potential as prognostic biomarkers of endometrial cancer*. *J Steroid Biochem Mol Biol*, 2018. 178: p. 303-311.
24. <sup>a</sup>Iizuka, N., et al., *Self-organizing-map-based molecular signature representing the development of hepatocellular carcinoma*. *FEBS Lett*, 2005. 579(5): p. 1089-100.
25. <sup>a, b, c</sup>Tsai, H.W., et al., *Progesterone receptor membrane component 1 as a potential prognostic biomarker for hepatocellular carcinoma*. *World J Gastroenterol*, 2018. 24(10): p. 1152-1166.
26. <sup>a</sup>Sun, B., et al., *Programmed cell death 10 promotes metastasis and epithelial-mesenchymal transition of hepatocellular carcinoma via PP2Ac-mediated YAP activation*. *Cell Death Dis*, 2021. 12(9): p. 849.
27. <sup>a, b, c, d</sup>Gao, Y.X., et al., *Progress and prospects of biomarkers in primary liver cancer (Review)*. *Int J Oncol*, 2020. 57(1): p. 54-66.
28. <sup>a</sup>Rojas, Á., et al., *Performance of different biomarkers for the management of hepatocellular carcinoma*. *Hepatoma Research*, 2018. 4(7): p. 31.
29. <sup>a</sup>Weinhold, N., et al., *Genome-wide analysis of noncoding regulatory mutations in cancer*. *Nat Genet*, 2014. 46(11): p. 1160-5.
30. <sup>a</sup>Hartmaier, R.J., et al., *High-Throughput Genomic Profiling of Adult Solid Tumors Reveals Novel Insights into Cancer Pathogenesis*. *Cancer Res*, 2017. 77(9): p. 2464-2475.
31. <sup>a</sup>Micheel, C.M., et al., *American Association for Cancer Research Project Genomics Evidence Neoplasia Information Exchange: From Inception to First Data Release and Beyond-Lessons Learned and Member Institutions' Perspectives*.



JCO Clin Cancer Inform, 2018. 2: p. 1-14.

32. <sup>a</sup> Consortium, A.P.G., AACR Project GENIE: Powering Precision Medicine through an International Consortium. *Cancer Discov*, 2017. 7(8): p. 818-831.
33. <sup>a</sup> Collins, F.S. and A.D. Barker, Mapping the cancer genome. Pinpointing the genes involved in cancer will help chart a new course across the complex landscape of human malignancies. *Sci Am*, 2007. 296(3): p. 50-7.
34. <sup>a</sup> Grossman, R.L., et al., Toward a Shared Vision for Cancer Genomic Data. *N Engl J Med*, 2016. 375(12): p. 1109-12.
35. <sup>a, b, c</sup> Goldman, M.J., et al., Visualizing and interpreting cancer genomics data via the Xena platform. *Nat Biotechnol*, 2020. 38(6): p. 675-678.
36. <sup>a, b, c, d</sup> Gyorffy, B., et al., An online survival analysis tool to rapidly assess the effect of 22,277 genes on breast cancer prognosis using microarray data of 1,809 patients. *Breast Cancer Res Treat*, 2010. 123(3): p. 725-31.
37. <sup>a</sup> Jiang, X., et al., Alternatively spliced isoforms reveal a novel type of PTB domain in CCM2 protein. *Sci Rep*, 2019. 9(1): p. 15808.
38. <sup>a</sup> Zhang, J., et al., Novel functions of CCM1 delimit the relationship of PTB/PH domains. *Biochim Biophys Acta Proteins Proteom*, 2017. 1865(10): p. 1274-1286.
39. <sup>a</sup> Abou-Fadel, J., et al., Comparative omics of CCM signaling complex (CSC). *Chin Neurosurg J*, 2020. 6: p. 4.
40. <sup>a</sup> Li, Y., et al., Integrated Bioinformatics Analysis Reveals Key Candidate Genes and Pathways Associated With Clinical Outcome in Hepatocellular Carcinoma. *Front Genet*, 2020. 11: p. 814.
41. <sup>a</sup> Zhang, G., et al., DPEP1 inhibits tumor cell invasiveness, enhances chemosensitivity and predicts clinical outcome in pancreatic ductal adenocarcinoma. *PLoS One*, 2012. 7(2): p. e31507.
42. <sup>a</sup> Hanahan, D. and R.A. Weinberg, Hallmarks of cancer: the next generation. *Cell*, 2011. 144(5): p. 646-74.
43. <sup>a</sup> Miranda, A., et al., Cancer stemness, intratumoral heterogeneity, and immune response across cancers. *Proc Natl Acad Sci U S A*, 2019. 116(18): p. 9020-9029.
44. <sup>a</sup> Batlle, E. and H. Clevers, Cancer stem cells revisited. *Nat Med*, 2017. 23(10): p. 1124-1134.
45. <sup>a</sup> Nassar, D. and C. Blanpain, Cancer Stem Cells: Basic Concepts and Therapeutic Implications. *Annu Rev Pathol*, 2016. 11: p. 47-76.
46. <sup>a, b</sup> Zheng, Q., et al., Immune signature-based hepatocellular carcinoma subtypes may provide novel insights into therapy and prognosis predictions. *Cancer Cell Int*, 2021. 21(1): p. 330.
47. <sup>a, b, c, d, e, f</sup> Thorsson, V., et al., The Immune Landscape of Cancer. *Immunity*, 2018. 48(4): p. 812-830 e14.
48. <sup>a</sup> Wang, W., et al., The clinical significance of microvascular invasion in the surgical planning and postoperative sequential treatment in hepatocellular carcinoma. *Sci Rep*, 2021. 11(1): p. 2415.
49. <sup>a</sup> Beaufreire, A., J. Calderaro, and V. Paradis, Combined hepatocellular-cholangiocarcinoma: An update. *J Hepatol*, 2021. 74(5): p. 1212-1224.
50. <sup>a</sup> Navarro-Imaz, H., Y. Rueda, and O. Fresnedo, SND1 overexpression deregulates cholesterol homeostasis in hepatocellular carcinoma. *Biochim Biophys Acta*, 2016. 1861(9 Pt A): p. 988-996.
51. <sup>a</sup> Wang, X., et al., Bile Acid Receptors and Liver Cancer. *Curr Pathobiol Rep*, 2013. 1(1): p. 29-35.
52. <sup>a</sup> Monte, M.J., et al., Bile acid secretion during rat liver carcinogenesis. *Life Sci*, 2000. 66(12): p. 1085-95.
53. <sup>a</sup> Wang, R., J.A. Sheps, and V. Ling, ABC transporters, bile acids, and inflammatory stress in liver cancer. *Curr Pharm*



*Biotechnol*, 2011. 12(4): p. 636-46.

54. <sup>^</sup>Zuo, Q., et al., *PPARgamma Coactivator-1alpha Suppresses Metastasis of Hepatocellular Carcinoma by Inhibiting Warburg Effect by PPARgamma-Dependent WNT/beta-Catenin/Pyruvate Dehydrogenase Kinase Isozyme 1 Axis*. *Hepatology*, 2021. 73(2): p. 644-660.
55. <sup>^</sup>Dimri, M. and A. Satyanarayana, *Molecular Signaling Pathways and Therapeutic Targets in Hepatocellular Carcinoma*. *Cancers (Basel)*, 2020. 12(2).
56. <sup>^</sup>Lou, J., et al., *Biomarkers for Hepatocellular Carcinoma*. *Biomark Cancer*, 2017. 9: p. 1-9.
57. <sup>a, b</sup>Parikh, N.D., et al., *Biomarkers for the Early Detection of Hepatocellular Carcinoma*. *Cancer Epidemiol Biomarkers Prev*, 2020. 29(12): p. 2495-2503.
58. <sup>^</sup>Tzartzeva, K., et al., *Surveillance Imaging and Alpha Fetoprotein for Early Detection of Hepatocellular Carcinoma in Patients With Cirrhosis: A Meta-analysis*. *Gastroenterology*, 2018. 154(6): p. 1706-1718 e1.
59. <sup>^</sup>Zhang, J., et al., *The threshold of alpha-fetoprotein (AFP) for the diagnosis of hepatocellular carcinoma: A systematic review and meta-analysis*. *PLoS One*, 2020. 15(2): p. e0228857.
60. <sup>^</sup>Nathavitharana, R.L., et al., *The use of AFP and DCP as serum biomarkers to differentiate between hepatocellular carcinoma and intrahepatic cholangiocarcinoma in non-cirrhotic livers*. *Journal of Clinical Oncology*, 2013. 31(15): p. e15162-e15162.
61. <sup>^</sup>Saffroy, R., et al., *New perspectives and strategy research biomarkers for hepatocellular carcinoma*. *Clin Chem Lab Med*, 2007. 45(9): p. 1169-79.
62. <sup>a, b</sup>Zhou, Y.M., et al., *Clinicopathologic characteristics of intrahepatic cholangiocarcinoma in patients with positive serum a-fetoprotein*. *World J Gastroenterol*, 2008. 14(14): p. 2251-4.
63. <sup>a, b</sup>Peluso, J.J., et al., *Regulation of ovarian cancer cell viability and sensitivity to cisplatin by progesterone receptor membrane component-1*. *J Clin Endocrinol Metab*, 2008. 93(5): p. 1592-9.
64. <sup>^</sup>Neubauer, H., et al., *Breast cancer proteomics reveals correlation between estrogen receptor status and differential phosphorylation of PGRMC1*. *Breast Cancer Res*, 2008. 10(5): p. R85.
65. <sup>^</sup>Pinter, M., et al., *Cancer and liver cirrhosis: implications on prognosis and management*. *ESMO Open*, 2016. 1(2): p. e000042.
66. <sup>^</sup>Tian, Z., et al., *Macrophages and hepatocellular carcinoma*. *Cell Biosci*, 2019. 9: p. 79.
67. <sup>^</sup>Roy, S., S. Glaser, and S. Chakraborty, *Inflammation and Progression of Cholangiocarcinoma: Role of Angiogenic and Lymphangiogenic Mechanisms*. *Front Med (Lausanne)*, 2019. 6: p. 293.
68. <sup>^</sup>Zhong, W., et al., *Mesenchymal stem cells in inflammatory microenvironment potently promote metastatic growth of cholangiocarcinoma via activating Akt/NF-kappaB signaling by paracrine CCL5*. *Oncotarget*, 2017. 8(43): p. 73693-73704.
69. <sup>^</sup>Yan, J., et al., *Prevalence and clinical relevance of T-helper cells, Th17 and Th1, in hepatitis B virus-related hepatocellular carcinoma*. *PLoS One*, 2014. 9(5): p. e96080.
70. <sup>^</sup>Shen, B., et al., *PPARgamma inhibits hepatocellular carcinoma metastases in vitro and in mice*. *Br J Cancer*, 2012. 106(9): p. 1486-94.
71. <sup>^</sup>Schaefer, K.L., et al., *Peroxisome proliferator-activated receptor gamma inhibition prevents adhesion to the*

extracellular matrix and induces anoikis in hepatocellular carcinoma cells. *Cancer Res*, 2005. 65(6): p. 2251-9.

72. ^ Dong, N., et al., M2 macrophages mediate sorafenib resistance by secreting HGF in a feed-forward manner in hepatocellular carcinoma. *Br J Cancer*, 2019. 121(1): p. 22-33.
73. ^ Hollenbach, M., The Role of Glyoxalase-I (Glo-I), Advanced Glycation Endproducts (AGEs), and Their Receptor (RAGE) in Chronic Liver Disease and Hepatocellular Carcinoma (HCC). *Int J Mol Sci*, 2017. 18(11).
74. ^ Lafdil, F., et al., Th17 cells and their associated cytokines in liver diseases. *Cell Mol Immunol*, 2010. 7(4): p. 250-4.
75. ^ Sequera, C., et al., How Rap and its GEFs control liver physiology and cancer development. C3G alterations in human hepatocarcinoma. *Hepat Oncol*, 2018. 5(1): p. HEP05.
76. ^ Carbajo-Pescador, S., et al., FoxO proteins: regulation and molecular targets in liver cancer. *Curr Med Chem*, 2014. 21(10): p. 1231-46.
77. ^ Cheng, X., et al., Up-regulation of chemokine receptor CCR4 is associated with Human Hepatocellular Carcinoma malignant behavior. *Sci Rep*, 2017. 7(1): p. 12362.
78. ^ Jang, K.Y., et al., SIRT1 and c-Myc promote liver tumor cell survival and predict poor survival of human hepatocellular carcinomas. *PLoS One*, 2012. 7(9): p. e45119.
79. ^ Qu, C., et al., Overexpressed PAQR4 predicts poor overall survival and construction of a prognostic nomogram based on PAQR family for hepatocellular carcinoma. *Math Biosci Eng*, 2022. 19(3): p. 3069-3090.