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ORIGINAL RESEARCH

Cross-Linked Multimeric Pro-Peptides of Type III Collagen (PC3X) in Hepatocellular Carcinoma – A Biomarker That Provides Additional Prognostic Value in AFP Positive Patients

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Purpose: Non-invasive biomarkers for diagnosing and prognosing hepatocellular carcinoma (HCC) are urgently needed. Cirrhosis is present in 80–90% of HCC patients. Cirrhosis is characterized by deposition and cross-linking of collagens that have crucial roles in HCC initiation and progression. We evaluated circulating cross-linked pro-peptides of type III collagen (PC3X) as a diagnostic and prognostic biomarker for HCC.

Patients and Methods: PC3X was measured by ELISA in plasma from patients with HCC (n=79), cirrhosis (n=86), non-cirrhotic hepatitis-B infection (n=74) and from healthy controls (n=44). PC3X was compared to the liver fibrosis marker PRO-C3 and the HCC tumor-cell derived marker alpha-fetoprotein (AFP). Diagnostic and prognostic potential was evaluated by AUROC and by calculating hazard ratios (HR) for progression-free survival (PFS) and overall survival (OS).

Results: PC3X, PRO-C3 and AFP were significantly elevated in patients with HCC compared to other liver diseases and healthy controls (p=0.0002, p<0.0001). In patients with normal AFP (<20 IU/mL), PC3X and PRO-C3 separated HCC from cirrhosis with an AUROC of 0.72 and 0.68, respectively. High PC3X and AFP predicted for poor PFS (HR_{PC3X}=1.80, p=0.032; HR_{AFP}=1.70, p=0.031) and OS (HR_{PC3X}=2.12, p=0.024; HR_{AFP}=2.55; p=0.003), whereas PRO-C3 did not (PFS: HR=1.19, p=0.059 and OS: HR=1.12, p=0.324). PC3X was independent of AFP (PFS: HR=1.74, p=0.045 and OS: HR=2.21, p=0.018) and combining the two improved prognostic value (PFS: HR=2.66, p=0.004 and OS: HR=5.86, p<0.0001).

Conclusion: PC3X is associated with HCC independent of AFP and provides diagnostic and prognostic value for HCC patients. If validated, this suggests that PC3X has biomarker potential for HCC.

Keywords: tumor microenvironment, extracellular matrix, fibrosis, liver cancer

Introduction

Hepatocellular carcinoma (HCC) remains a major global health problem, and in contrast to most other types of cancer, its incidence has increased over the last decades.[1] HCC is highly lethal and the 5-year survival rate is approximately 10%.[2,3] This poor prognosis is due to both late-stage diagnosis and limited treatment options.

Currently, there is compelling evidence that earlier stage detection of HCC via surveillance is more amenable to curative therapies and improved overall survival

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(OS).[4] Though all current guidelines recommend surveillance for HCC in patients with cirrhosis, the recommended approaches are not consistent. While the European Association for the Study of the Liver (EASL) recommends the use of ultrasonography alone,[5] the American Association for the Study of Liver Diseases (AASLD) recommends the use of ultrasonography with or without alpha-fetoprotein (AFP)[6] and the Asian Pacific Association for the Study of the Liver (APASL) recommends the use of both ultrasonography and AFP.[7] Though AFP is the gold-standard serum marker for HCC, the utility of AFP for surveillance is limited by its low sensitivity and specificity.[8] A large proportion of HCC patients (42%) in fact do not have elevated levels of AFP (>20 IU/mL),[9] emphasizing the urgent need to develop better biomarkers.

Cirrhosis, a consequence of fibroblast activation leading to extracellular matrix (ECM) deposition is the major risk factor for HCC and is the substrate on which tumors develop in 80–90% of cases.[10] Cross-linking of the ECM is important as it contributes to tissue stiffness thereby changing its quality. Mounting evidence also suggests that ECM deposition and stiffness play key roles in HCC initiation, progression and metastasis.[11,12] Cross-linking of collagens is catalyzed by enzymes such as lysyl oxidase (LOX), its family members LOX-like (LOXL) 1–4 and transglutaminase 2 (TG2).[13,14] LOXL2-induced tissue stiffness has been shown to induce intrahepatic and extrahepatic metastasis in HCC, while TG2 is elevated in HCC patients and is associated with HCC invasion.[15–17]

Type III collagen has been detected in HCC patients in a study from 1997.[18] In liver fibrosis, type III collagen production is markedly up-regulated.[19] During its formation and accumulation, pro-peptides are cleaved from type III pro-collagen and released into the circulation. Notably, we have shown that the biomarker PRO-C3, which targets the N-proteinase ADAMTS2 cleavage site of the N-terminal pro-peptide of type III collagen and consequently measures true formation of type III collagen, is a robust biomarker of liver fibrosis (Figure 1). PRO-C3 has been widely investigated as a diagnostic, prognostic, and efficacy biomarker, as well as a predictor of clinical outcome across different liver diseases.[19–26] Elevated PRO-C3 levels have furthermore

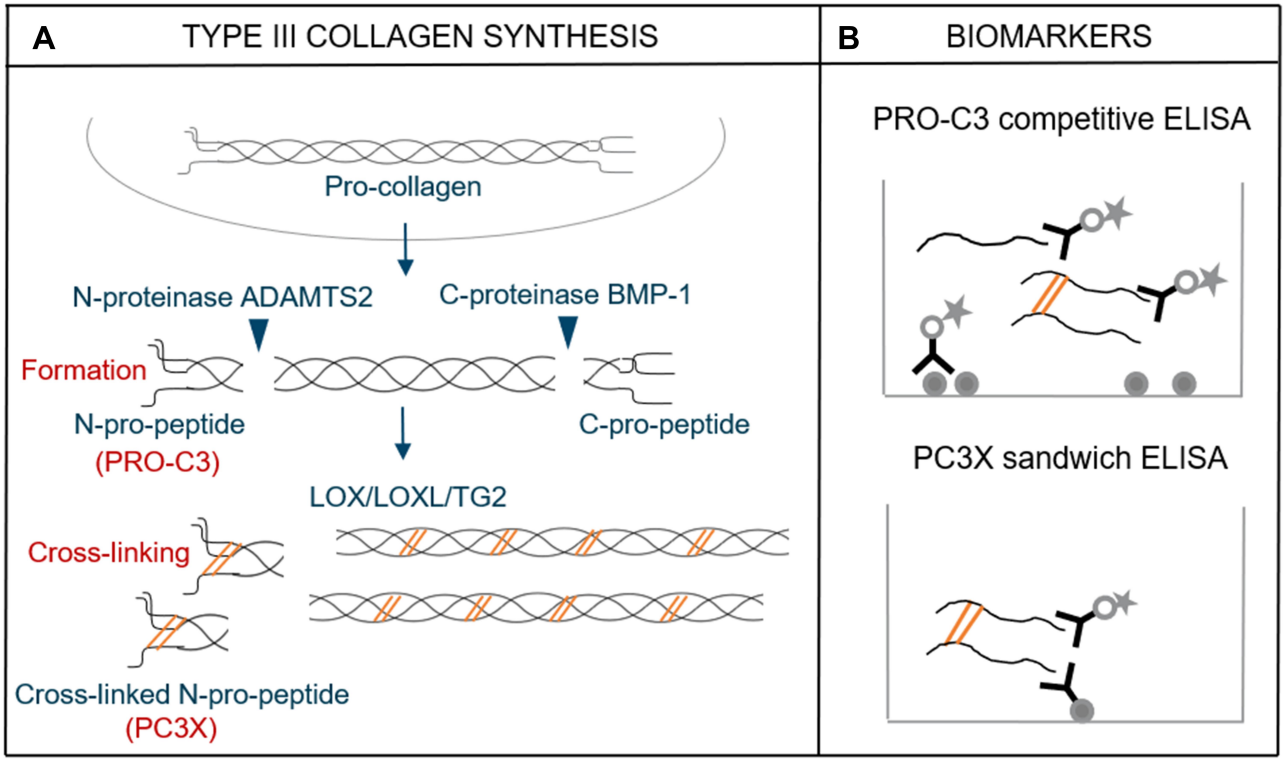

Figure 1 Biomarkers of type III collagen formation and cross-linking. (**A**) After pro-collagen triple helix folding (α1 chains), the N-and C-pro-peptides are cleaved in the extracellular space by the N-proteinase ADAMTS2 and the C-proteinase BMP-1. Type III collagen molecules are cross-linked by the enzymes lysyl oxidase (LOX), LOX-like (LOXL) 1–4 and transglutaminase 2 (TG2). (**B**) PRO-C3 measures type III collagen formation. PRO-C3 is based on a competitive ELISA that targets the ADAMTS2 cleavage site of the N-terminal pro-peptide and can measure single-and doublet stranded pro-peptides. PC3X measures type III collagen formation and cross-linking. PC3X is based on a sandwich ELISA that targets the same cleavage site as PRO-C3 but only measures cross-linked multimeric pro-peptides.

been associated with poor outcomes in breast cancer and malignant melanoma.[27,28] However, PRO-C3's role in HCC remains to be established. The PRO-C3 biomarker does not differentiate between single stranded or cross-linked N-terminal pro-peptides (Figure 1). Because liver fibrosis progression and HCC growth and metastasis are associated with matrix stiffness and cross-linking of collagens,[11,15–17,29] we hypothesized that an ECM derived biomarker only measuring cross-linked type III collagen pro-peptides would have diagnostic and prognostic potential for HCC. To investigate this, we developed a sandwich ELISA to measure circulating cross-linked multimeric pro-peptides of type III collagen (PC3X) (Figure 1) and evaluated its biomarker potential in HCC in comparison with PRO-C3 and AFP.

Patients and Methods

Patient Cohort

This case–control study involved four independent groups comprising a total of 283 participants (HCC (n=79), cirrhosis (n=86), non-cirrhotic hepatitis B virus (HBV) infection (n=74) and healthy controls (n=44)) recruited from a single tertiary liver clinic in Sydney, Australia. The HCC patients had different etiologies and were diagnosed by characteristic radiological appearances on 4-phase CT or MRI demonstrating the combination of hypervascularity in late arterial phase (enhancement) and washout on the portal venous and/or delayed phases according to EASL guidelines,[5] or by histology. The absence of HCC was based on imaging evidence on the absence of space-occupying lesions in the liver consistent with HCC on serial imaging every 6 months for the preceding 12 months. Clinical staging of HCC was according to the Barcelona Clinic Liver Cancer (BCLC) system. EDTA plasma was taken at the time of diagnosis, prior to the initiation of treatment. The HCC patients received different therapeutic options: best supportive care (n=3), radiofrequency ablation (n=15), selective internal radiation therapy (Sirspheres) (n=5), sorafenib (n=14), surgical (n=11) and transarterial chemoembolization (TACE) (n=31). Progression-free survival (PFS) and OS were estimated from baseline.

The cirrhosis group comprised individuals with different etiologies, diagnosed based on liver biopsy, a fibroscan value of greater than 14 kPa, or clinical feature suggestive of cirrhosis (presence of varices, splenomegaly or thrombocytopenia). The HBV infected group included patients with chronic HBV in the absence of cirrhosis. The healthy control group comprised individuals recruited through advertisements in local newspapers and at the hospital. All had normal physical examinations and liver tests, negative viral hepatitis serology and no history of liver disease. The study protocol was approved by the Human Ethics Committee of the Sydney West Area Health Service (HREC No.2002/12/4.9 (1564)) in compliance with the Helsinki Declaration. Written informed consent was obtained from all participants.

Clinical and Laboratory Data

Demographic and clinical data, including age, gender, body mass index (BMI), etiology (HCV, HBV, alcoholic liver disease, non-alcoholic steatohepatitis), liver parameters (diabetes status, levels of bilirubin, albumin, alanine transaminase (ALT), aspartate transaminase (AST), platelet count (PLT) and AFP) and tumor-related variables (BCLC stage, Child-Pugh score, size of largest lesion, number of lesions, existence of metastasis and existence of portal vein invasion) are shown in Table 1. Routine biochemical tests including bilirubin, albumin, ALT, AST, PLT and AFP were assessed in fasting blood samples by standard methods and assays at baseline.

ELISA Measurements – Type III Collagen

A monoclonal antibody was raised against the N-proteinase generated neo-epitope of the N-terminal pro-peptide of type III collagen and used to develop a technically robust sandwich ELISA based on two monoclonal antibodies (PC3X) (see Text, Supplemental Digital Content 1, which includes detailed methods about PC3X assay development, procedure and technical evaluation) and (see Text and Table in Supplemental Digital Content 2, which show the technical evaluation of the PC3X assay).

The procedure in brief, streptavidin-coated microtiter plates were coated with a biotin-labeled catcher antibody specific towards the N-proteinase cleavage site of the N-terminal pro-peptide of type III collagen and incubated for 30 minutes at 20°C. Standard, controls or pre-diluted EDTA plasma sample were added followed immediately by addition of assay buffer and incubated for 20 hours at 4°C. Then, horseradish peroxidase (HRP)-labeled detector antibody was added and incubated for 1 hour at 20°C. Next, tetramethylbenzidine (TMB) was added and incubated for 15 minutes at 20°C. All incubations included shaking of the plates followed by five times washing. To stop the reaction of TMB, sulfuric acid was added, and the

Table 1 Patient Characteristics: Liver Diseases and Healthy Controls

n	Healthy Controls	Non-Cirrhotic HBV	Cirrhosis	HCC	P-value
	44	74	86	79	
Age (years), mean ± SD	53.8 (7.6)	58.6 (9.1)	58.8 (10.0)	62.0 (11.6)	0.0004
Gender (male), n (%)	41 (93.2)	63 (85.1)	75 (87.2)	71 (89.9)	0.567
BMI, mean ± SD	25.9 (2.9)	25.6 (4.2)	29.4 (5.6)	28.1 (6.0)	<0.0001
Etiology					
HCV, n (%)	n/a	0 (0)	43 (50.0)	38 (48.1)	<0.0001
HBV, n (%)	n/a	74 (100)	23 (26.7)	13 (16.5)	
EtOH, n (%)	n/a	0 (0)	7 (8.1)	10 (12.7)	
NASH, n (%)	n/a	0 (0)	10 (11.6)	14 (17.7)	
Other, n (%)	n/a	0 (0)	3 (3.5)	4 (5.1)	
Ethnicity					
Caucasian, n (%)	33 (75.0)	8 (10.8)	49 (57.0)	50 (63.3)	
Chinese, n (%)	6 (13.6)	48 (64.9)	12 (14.0)	12 (15.2)	
Middle eastern, n (%)	1 (2.3)	9 (12.2)	20 (23.3)	9 (11.4)	<0.0001
Indian, n (%)	4 (9.1)	5 (6.8)	3 (3.5)	3 (3.8)	
African, n (%)	0 (0)	2 (2.7)	1 (1.2)	3 (3.8)	
Polynesian, n (%)	0 (0)	2 (2.7)	1 (1.2)	2 (2.5)	
Diabetics, n (%)	0 (0)	10 (13.5)	29 (33.7)	30 (38.0)	<0.0001
Bilirubin, mean ± SD	13.8 (5.4)	13.5 (8.4)	21.2 (14.5)	22.0 (24.0)	<0.0001
Albumin, mean ± SD	43.6 (1.8)	43.6 (3.0)	40.5 (5.3)	36.6 (6.7)	<0.0001
ALT, mean ± SD	30.8 (10.9)	41.5 (38.5)	65.3 (61.5)	86.9 (89.4)	<0.0001
AST, mean ± SD	28.9 (7.3)	40.6 (13.4)	75.0 (58.2)	111.8 (101.3)	<0.0001
PLT, mean ± SD	231.6 (52.2)	227.3 (52.6)	131.6 (66.0)	126.6 (64.0)	<0.0001
AFP (IU/mL), mean ± SD	n/a	2.6 (1.0)	6.5 (12.3)	1869.6 (10572.0)	<0.0001
BCLC staging, 0/A/B/C/D	n/a	n/a	n/a	4/29/30/13/3	
Child-Pugh score, A/B/C/n/a	n/a	n/a	78/6/2/0	50/13/7/9	
Size of largest lesion, mean ±SD	n/a	n/a	n/a	4.5 (3.9)	
Number of lesions, mean ± SD	n/a	n/a	n/a	2.5 (2.6)	
Metastasis, Y/N	n/a	n/a	n/a	6/73	
Portal vein invasion, Y/N	n/a	n/a	n/a	11/68	

Notes: Results are expressed as mean (standard deviation) or frequency (percentage); P values were calculated using Kruskal–Wallis test with Dunn's multiple comparisons or a chi-square test.

Abbreviations: HCC, hepatocellular carcinoma; BMI, body mass index; HCV, hepatitis C virus; HBV, hepatitis B virus; EtOH, alcoholic liver disease; NASH, non-alcoholic steatohepatitis; ALT, alanine transaminase; AST, aspartate transaminase; PLT, platelet count; AFP, alpha-fetoprotein; BCLC, Barcelona Clinic Liver Cancer.

plates were analyzed in a VersaMax ELISA microplate reader at 450 nm with 650 nm as reference.

PRO-C3 was assessed in EDTA plasma samples for comparison to PC3X. The PRO-C3 competitive ELISA is a well-characterized assay based on a monoclonal antibody specific towards the N-proteinase cleavage site of the N-terminal pro-peptide of type III collagen manufactured by Nordic Bioscience (Herlev, Denmark) and performed according to the manufacturer's specifications.[30]

Statistical Analyses

Characteristics of the patient groups are presented as frequency (percentage) for categorical variables and mean (standard deviation) for continuous variables. Statistical differences for categorical variables were assessed using chi-square tests whereas differences for continuous variables were evaluated using the Kruskal–Wallis test. Differences between biomarker levels in the different patient groups were assessed using the Kruskal–Wallis test adjusted for Dunn's multiple comparisons test. Pearson's correlation analysis was performed to describe the relationship between PC3X and PRO-C3 levels. The diagnostic power of the biomarkers was investigated by the area under the receiver operating characteristics (AUROC) curve and calculated using the method of Delong et al.[31] The association between biomarker levels

and clinical variables was assessed by multiple regression analysis using the enter method. Kaplan–Meier (KM) survival curves were used to analyze PFS and OS and a Log rank test was used to determine differences between KM curves. A Cox proportional-hazards regression model was used to calculate the hazard ratios (HR) with 95% CI for prediction of OS and PFS for the biomarkers and other clinical covariates: age, gender, BMI, Child-Pugh score, number of lesions, maximal tumor size and presence/absence of portal vein invasion. Multivariate Cox proportional-hazards regression was used to assess the independent predictive value of PC3X and AFP adjusted for the above mentioned clinical variables. For statistical purposes, PC3X and PRO-C3 levels under the 75th percentile cut-point were used as a reference to calculate the HR for patients with levels above the 75th percentile, which was based on the data distribution and previous studies.[27,28] AFP levels under 20 IU/mL were used as a reference to calculate the HR for patients with elevated AFP levels.[32] Sample size calculation was performed to calculate the adequate sample size for comparing PC3X biomarker levels in patients with HCC to PC3X levels in patients with cirrhosis (alpha: 0.05, power: 0.8, difference of means: 7, standard deviation (SD): 14), resulting in a calculated sample size of 64 patients in each group. Another sample size calculation was performed to calculate the adequate sample size for conducting the survival analysis of PC3X (alpha: 0.05, power: 0.8, survival group 0 and 1: 0.70 and 0.35, ratio of sample sizes in group 1/group 2: 4), resulting in a calculated sample size of 69 patients in total.

Statistical analyses were performed using MedCalc (v16.8.4) and Graphpad Prism (v7.01) (GraphPad Software, CA, USA). A P value < 0.05 was considered statistically significant.

Results

Patient Characteristics

The clinical characteristics of the patients with HCC, cirrhosis, non-cirrhotic HBV infection and healthy controls are summarized in Table 1. Some differences were observed between the groups and minor differences were seen according to age. Patients with cirrhosis/HCC had a greater BMI compared to the healthy controls and non-cirrhotic HBV infected patients. Most of the patients were males, but no significant difference was observed between the different groups of patients. No differences were seen in underlying etiology when comparing patients with cirrhosis and HCC. When evaluating ethnicity, the majority of patients in the non-cirrhotic HBV infected group were Chinese whereas most of the patients in the other groups were Caucasian. When assessing liver parameters, statistically significant differences were found in diabetes status, and levels of bilirubin, albumin, ALT, AST, PLT and AFP. Most of the HCC patients had Child-Pugh A disease and stage A or B according to BCLC staging.

Plasma PC3X, PRO-C3 and AFP Levels Were Elevated in Patients with HCC

PC3X, PRO-C3 and AFP were measured in healthy controls and patients with non-cirrhotic HBV infection, cirrhosis and HCC at the time of diagnosis, prior to the initiation of treatment (Figure 2A–C). PC3X, PRO-C3 and AFP levels were significantly elevated in HCC patients compared to those with cirrhosis (PC3X: p=0.0002, PRO-C3: p=0.0016, AFP: p<0.0001), non-cirrhotic HBV infection (p<0.0001) and healthy controls (PC3X and PRO-C3: p<0.0001, AFP: N/A). Of note, 58% of the HCC patients had normal AFP levels (<20 IU/mL). PRO-C3 was significantly higher in patients with non-cirrhotic HBV infection compared to healthy controls (p=0.040), whereas PC3X was not (p>0.999). In addition, the level of PC3X, PRO-C3 and AFP was significantly elevated in cirrhosis compared to patients with non-cirrhotic HBV infection (PC3X: p=0.0013, PRO-C3: p<0.0001, AFP: p=0.018).

Next, the biomarker levels in cirrhosis and HCC patients were evaluated according to Child-Pugh score (Figure 2D–F). Plasma PC3X and PRO-C3 were significantly higher in Child-Pugh C compared to Child-Pugh A (PC3X: p=0.034, PRO-C3 : p=0.035), whereas no significant difference was observed in AFP (p>0.999). When biomarker levels in the HCC patients were evaluated according to BCLC staging, no significant differences between stages were observed in either PC3X, PRO-C3 or AFP (data not shown).

Association Between PC3X and PRO-C3

As PC3X and PRO-C3 reflect similar biology, we investigated the association between PC3X and PRO-C3 in patients without cirrhosis (healthy controls and patients with non-cirrhotic HBV infection) and with cirrhosis (cirrhosis and HCC patients). PC3X and PRO-C3 did not correlate in healthy controls and patients with non-cirrhotic HBV infection (r=0.06, p=0.506) (Figure 3A), whereas the two markers significantly correlated in patients with cirrhosis and HCC (r=0.53, p<0.0001) (Figure 3B).

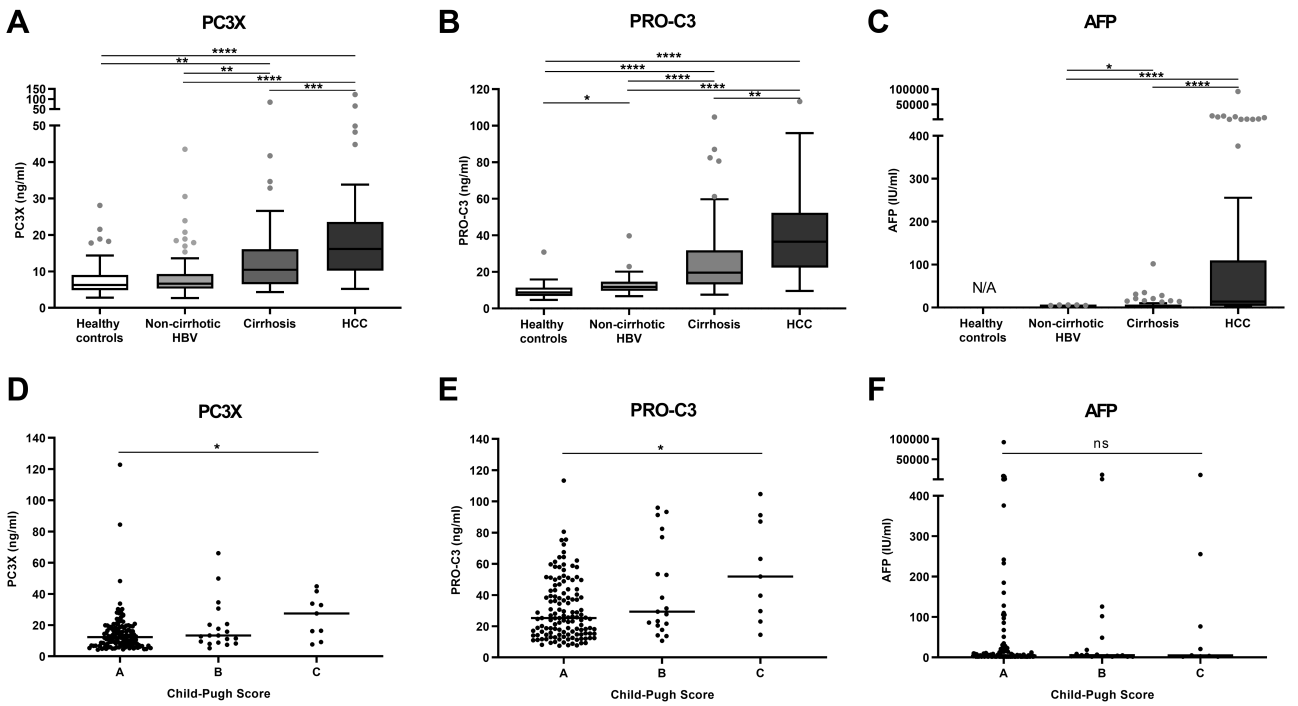

Figure 2 Evaluation of PC3X, PRO-C3 and AFP in healthy controls and in patients with non-cirrhotic HBV infection, cirrhosis and hepatocellular carcinoma (HCC). PC3X (**A**), PRO-C3 (**B**) and AFP levels (**C**) in healthy controls (n=44) and in patients with non-cirrhotic HBV infection (n=74), cirrhosis (n=86) and HCC (n=79). Data are presented as Tukey box plots. PC3X (**D**), PRO-C3 (**E**) and AFP (**F**) levels in cirrhosis and HCC patients separated by Child-Pugh score A (n=128), B (n=19) and C (n=9). The black horizontal lines represent the median value. Statistical differences were analyzed using the Kruskal–Wallis test adjusted for Dunn's multiple comparisons test (**A**–**F**). *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.
Abbreviation: ns, non-significant.

Similar Diagnostic Performance of PC3X, PRO-C3 and AFP

To evaluate the capability of separating patients with early HCC (BCLC stage 0 and A) from cirrhosis, the AUROC was used to assess the diagnostic performance of PC3X, PRO-C3 and AFP, individually. PC3X, PRO-C3 and AFP were able to separate patients with early HCC from cirrhosis without HCC with an AUROC of 0.70, 0.68 and 0.75, with sensitivities of

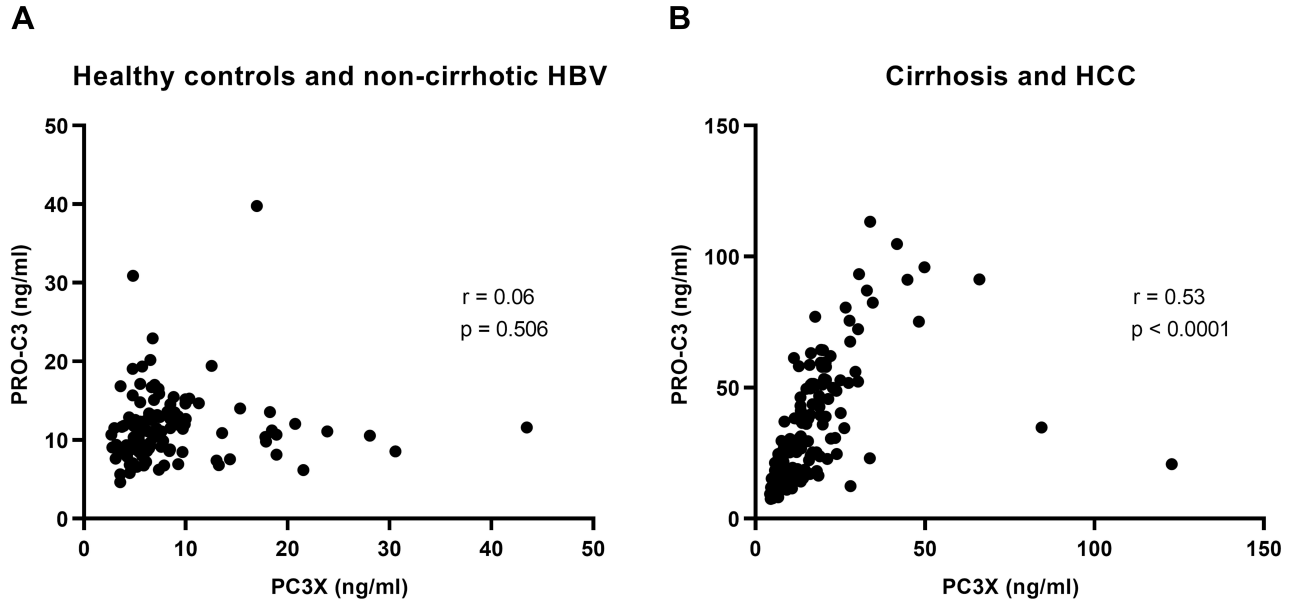

Figure 3 Correlation between PC3X and PRO-C3 levels. Pearson's correlation analysis was performed to describe the relationship between PC3X and PRO-C3 levels in EDTA plasma from healthy controls and patients with non-cirrhotic HBV infection (**A**), and in cirrhosis and HCC patients (**B**).

45.5%, 69.7% and 33.3% and specificities of 87.2%, 72.1% and 93.0%, respectively (Table 2) (see Table 1 in Supplemental Digital Content 3, which shows the ROC curves). Next, we evaluated PC3X and PRO-C3's diagnostic potential in patients with normal AFP (<20 IU/mL) and detected AUROCs of 0.72 and 0.68 for separating early HCC from cirrhosis.

Association of Biomarker Levels in HCC with Clinical Variables

We evaluated the correlation between PC3X, PRO-C3 and AFP levels in HCC patients with the liver function markers albumin, ALT, AST, bilirubin and PLT (see Table 1 in Supplemental Digital Content 4). PC3X correlated weakly to AFP (r=0.25, p=0.026), ALT (r=0.37, p=0.0009), AST (r=0.38, p=0.0006) and age (r=−0.41, p=0.0001) and strongly to PRO-C3 (r=0.72, p<0.0001). A weak correlation was also observed between PRO-C3 and AFP (r=0.30, p=0.007), age (r=−0.35, p=0.002), albumin (r=−0.26, p=0.022), ALT (r=0.38, p=0.0006) and AST (r=0.48, p<0.0001). AFP correlated weakly to ALT (r=0.29, p=0.009) and AST (r=0.29, p=0.009) in addition to PC3X and PRO-C3.

Next, we examined the association between PC3X, PRO-C3 and AFP levels in the HCC patients with the tumor-related clinical variables Child-Pugh score, size of largest lesion, number of lesions, existence of metastases and existence of portal vein invasion using a multiple regression analysis. The only clinical co-variates that contributed significantly to PC3X were the number of lesions (p=0.001), whereas the presence of portal vein invasion contributed to AFP (p=0.019) and no associations were found between PRO-C3 and these clinical variables (see Table 2 in Supplemental Digital Content 4, which shows the multiple regression analysis).

Prognostic Performance of PC3X, PRO-C3 and AFP in Patients with HCC

Next, we evaluated the prognostic potential of PC3X, PRO-C3 and AFP in HCC by Kaplan–Meier curves and Cox proportional-hazard models. The median PFS for HCC patients was 270 days (range, 30–3960 days) while the median OS was 1080 days (range, 30–6120 days). Using the Kaplan–Meier method, we assessed the association between biomarker levels and survival (Figure 4). High PC3X levels (>75th percentile) were associated with shorter PFS (p=0.024) and OS (p=0.011), compared to lower levels (≤75th percentile) (Figure 4A and D). The median PFS and OS were 180 and 540 days in biomarker high, versus 300 and 1530 days in biomarker low patients, for PFS and OS, respectively. High levels (>75th percentile) of the biomarker PRO-C3 were not associated with shorter PFS (p=0.054) or OS (p=0.383) compared to lower levels (≤75th percentile) (Figure 4B and E). High AFP (>20 IU/mL) was significantly associated with poor PFS (p=0.035) and OS (p=0.001) compared to lower levels (≤20 IU/mL) (Figure 4C and F). When combining PC3X and AFP, high PC3X and high AFP were associated with poor PFS (p=0.003) and OS (p<0.0001) compared to low levels (Figure 5). In detail, the median PFS and OS were 120 and 240 days for patients with high PC3X and high AFP levels, 255 and 960 days for patients with either high PC3X or high AFP, and 390 and 1920 days for patients with both low PC3X and low AFP, respectively.

The ability of PC3X to predict PFS and OS in HCC patients was then evaluated by Cox proportional-hazard models (Table 3). The patients with high PC3X levels (>75th percentile) had significantly poorer PFS and OS compared to those with lower PC3X levels (PFS:

Table 2 Discriminative Performance of Biomarkers in HCC with BCLC 0/A Vs Cirrhosis

HCC vs Cirrhosis	Cutoff Value (ng/mL)	Sensitivity (%)	Specificity (%)	PPV	NPV	+LHR	AUROC (95% CI)	P-value
PC3X	19.2	45.5	87.2	57.7	80.6	3.55	0.70 (0.61–0.78)	0.0002
PRO-C3	28.1	69.7	72.1	48.9	86.1	2.50	0.68 (0.59–0.76)	0.001
AFP	20.0	33.3	93.0	64.6	78.4	4.78	0.75 (0.67–0.83)	<0.0001
HCC vs cirrhotic patients with AFP <20 IU/mL								
PC3X	19.2	54.6	90.0	60.0	87.8	5.45	0.72 (0.62–0.81)	0.0009
PRO-C3	29.0	63.6	77.5	43.8	88.6	2.83	0.68 (0.58–0.77)	0.010

Notes: AUROC and p values were calculated using the method of Delong et al;[31] The cutoff value for PC3X and PRO-C3 was obtained from AUROC, whereas 20 UI/mL for AFP is an indication of elevated levels.[32]

Abbreviations: HCC, hepatocellular carcinoma; AFP, alpha-fetoprotein; PPV, positive predictive value; NPV, negative predictive value; LHR, likelihood ratio; AUROC, area under the receiver operating characteristics.

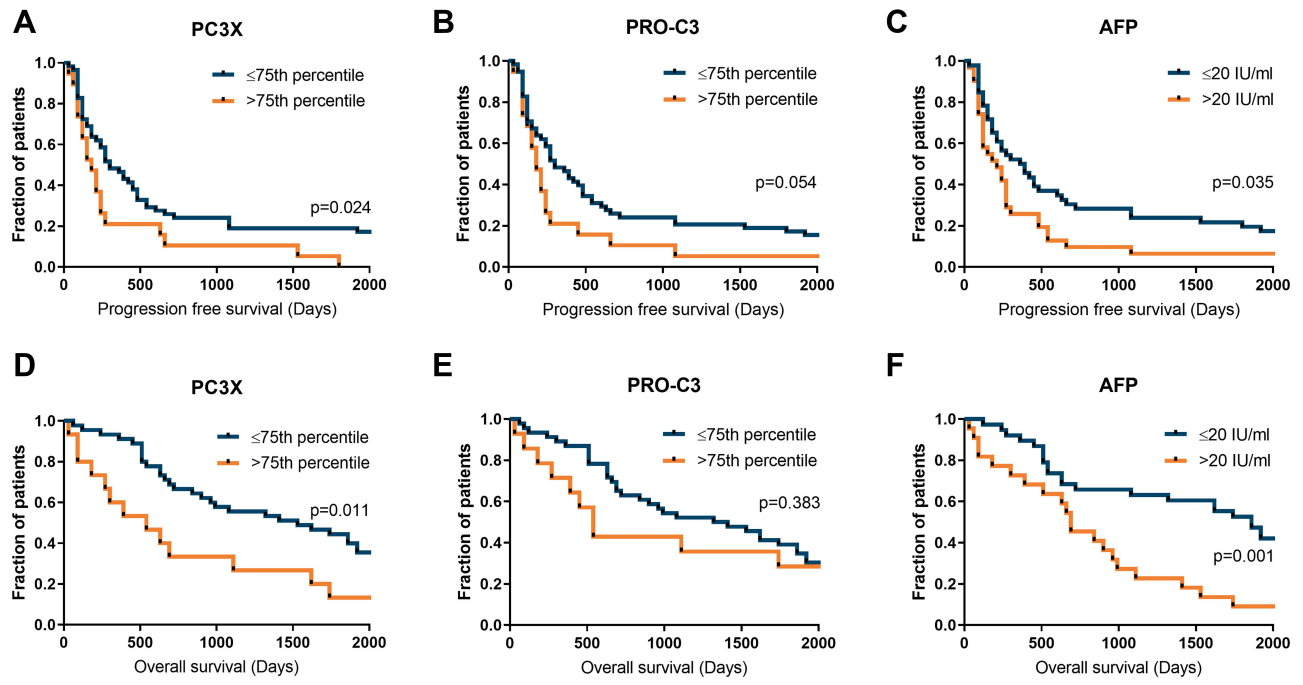

Figure 4 Kaplan–Meier analysis of progression-free survival and overall survival in hepatocellular carcinoma patients. Progression-free survival (**A**–**C**) and overall survival (**D**–**F**) for hepatocellular carcinoma patients with biomarker levels above the 75th percentile vs below for PC3X (**A** and **D**) and PRO-C3 (**B** and **E**), while for AFP it is above 20 UI/mL vs below (**C** and **F**). A Log rank test was used to determine differences between the curves. A p-value of p<0.05 was considered significant.

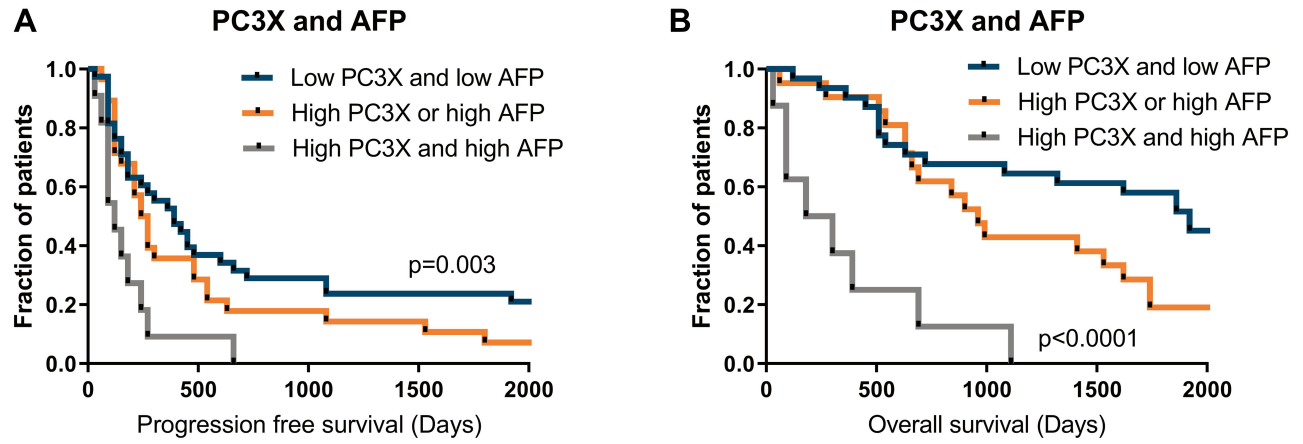

Figure 5 Progression-free survival and overall survival by Kaplan–Meier analysis, comparing the subgroups of patients with low or high PC3X and AFP levels in hepatocellular carcinoma. Progression-free survival (**A**) and overall survival (**B**) for hepatocellular carcinoma patients with either low PC3X and low AFP, high PC3X or high AFP, or high PC3X and high AFP. A Log rank test was used to determine differences between the curves. A p-value of p<0.05 was considered significant.

HR=1.80, 95% CI=1.05–3.08, *p*=0.032 and OS: HR=2.12, 95% CI=1.10–4.05, *p*=0.024). A similar trend was observed when PC3X was evaluated on a continuous univariate scale; PC3X was predictive of poor PFS (HR=1.02, 95% CI=1.01–1.04, *p*=0.007) and borderline predictive of poor OS (HR=1.03, 95% CI=1.00–1.06, *p*=0.054).

Comparable results were observed for AFP, high levels (>20 IU/mL) were predictive of poor PFS (HR=1.70, 95% CI=1.05–2.76, *p*=0.031) and OS (HR=2.55, 95% CI=1.38–4.69, *p*=0.003), and on a continuous univariate scale, AFP was predictive of poor OS (HR=1.00, 95% CI=1.00–1.00, *p*=0.030). In contrast, high PRO-C3 (>75th percentile) was not predictive of poor PFS (HR=1.19, 95% CI=0.99–1.42, *p*=0.059) or OS (HR=1.12, 95% CI=0.89–1.41, *p*=0.324). When combining PC3X and AFP, high PC3X and high AFP were predictive of PFS (HR=2.66, 95% CI=1.37-5-18, *p*=0.004) and OS (HR=5.86, 95% CI=2.57–13.37, *p*<0.0001) compared to low levels.

Table 3 Association Between Biomarker Levels, Clinical Covariates and Outcome for HCC Patients

Variables		Progression-Free Survival			Overall Survival		
Univariate Analysis		**HR**	**95% CI**	**P-value**	**HR**	**95% CI**	**P-value**
Age	Continuous	1.01	0.99–1.03	0.332	1.01	0.99–1.04	0.280
Gender (male)	Male vs female	0.78	0.36–1.71	0.533	1.04	0.41–2.65	0.932
BMI	Continuous	1.01	0.97–1.05	0.672	0.99	0.94–1.04	0.634
Child-Pugh score	B/C vs A	2.39	1.37–4.19	0.002	5.06	2.58–9.93	<0.0001
Size of largest lesion	Continuous	1.08	1.02–1.14	0.007	1.14	1.07–1.22	0.0001
Number of lesions	Continuous	1.18	1.08–1.29	0.0003	1.41	1.22–1.63	<0.0001
Portal vein invasion	Yes vs no	2.57	1.25–5.11	0.010	3.53	1.52–8.21	0.003
PC3X	Continuous	1.02	1.01–1.04	0.007	1.03	1.00–1.06	0.054
	High (23.9–122.8 ng/mL, Q4) vs low (5.2–23.5 ng/mL, Q1-Q3)	1.80	1.05–3.08	0.032	2.12	1.10–4.05	0.024
PRO-C3	Continuous	1.01	1.00–1.03	0.020	1.01	0.99–1.03	0.052
	High (52.3–113.3 ng/mL, Q4) vs low (5.3–51.8 ng/mL, Q1-Q3)	1.19	0.99–1.42	0.059	1.12	0.89–1.41	0.324
AFP	Continuous	1.00	1.00–1.00	0.171	1.00	1.00–1.00	0.030
	High vs low (≥20 vs <20 IU/mL)	1.70	1.05–2.76	0.031	2.55	1.38–4.69	0.003
PC3X and AFP	High PC3X and high AFP vs low PC3X and/or low AFP	2.66	1.37–5.18	0.004	5.86	2.57–13.37	<0.0001
Multivariate Analysis		**HR**	**95% CI**	**P-value**	**HR**	**95% CI**	**P-value**
Adjusted for age, gender and BMI							
PC3X	High vs low (Q4 vs Q1-Q3)	1.88	1.07–3.31	0.028	2.54	1.27–5.08	0.008
AFP	High vs low (≥20 vs <20 IU/mL)	1.64	0.99–2.73	0.054	2.50	1.31–4.75	0.005
PC3X and AFP	High PC3X and high AFP vs low PC3X and/or low AFP	2.81	1.38–5.74	0.004	6.35	2.66–15.15	<0.0001
Adjusted for Child-Pugh score							
PC3X	High vs low (Q4 vs Q1-Q3)	1.53	0.87–2.67	0.140	2.23	1.12–4.47	0.023
AFP	High vs low (≥20 vs <20 IU/mL)	1.96	1.17–3.29	0.011	4.53	2.24–9.19	<0.0001
PC3X and AFP	High PC3X and high AFP vs low PC3X and/or low AFP	2.68	1.36–5.28	0.004	8.17	3.31–20.13	<0.0001
Adjusted for size of largest lesion, number of lesions and presence of portal vein invasion							
PC3X	High vs low (Q4 vs Q1-Q3)	1.43	0.79–2.59	0.241	1.47	0.69–3.13	0.317
AFP	High vs low (≥20 vs <20 IU/mL)	1.65	0.98–2.76	0.059	2.26	1.12–4.56	0.023
PC3X and AFP	High PC3X and high AFP vs low PC3X and/or low AFP	2.01	0.99–4.07	0.053	4.40	1.77–10.89	0.001
PC3X adjusted for AFP	High vs low (Q4 vs Q1-Q3)	1.74	1.01–2.98	0.045	2.21	1.15–4.27	0.018

Notes: Hazard ratios were calculated by univariate and multivariate Cox proportional-hazard analysis; By univariate analysis, PC3X and PRO-C3 were analyzed on both a continuous scale and divided into quartiles with the lower levels (Q1-Q3) used as a reference to calculate the HR for patients in the upper quartile (Q4); The covariates were analyzed on a continuous scale, and Child-Pugh score and AFP were furthermore analyzed on a binominal scale; By multivariable analysis, PC3X and AFP were adjusted as indicated in the text.

Abbreviations: AFP, alpha-fetoprotein; BMI, body mass index; HR, hazard ratio.

To determine the independent predictive value of high PC3X, multivariate Cox proportional-hazard analysis was conducted. When PC3X was adjusted for the covariates age, gender and BMI, high PC3X was independently predictive of poor PFS and OS (PFS: HR=1.88, 95% CI=1.07–3.31, p=0.028 and OS: HR=2.54, 95% CI=1.27–5.08, p=0.008). PC3X was then adjusted for Child-Pugh score and the tumor-related variables size of

largest lesion, number of lesions and presence of portal vein invasion because these factors are individually predictive of poor PFS and OS (Table 3). PC3X was independently predictive of poor OS when adjusted for Child-Pugh score (HR=2.23, 95% CI=1.12–4.47, p=0.023) but not PFS; PC3X remained not significant when adjusted for size of largest lesion, number of lesions and presence of portal vein invasion. On the other hand, high AFP was predictive of poor PFS and OS when adjusted for Child-Pugh score (PFS: HR=1.96, 95% CI=1.17–3.29, p=0.011 and OS: HR=4.53, 95% CI=2.24–9.19, p<0.0001) and size of largest lesion, number of lesions and presence of portal vein invasion (PFS: HR=1.65, 95% CI=0.98–2.76, p=0.059 and OS: HR=2.26, 95% CI=1.12–4.56, p=0.023), though only borderline statistically significant of PFS. High AFP was predictive of poor OS when adjusted for age, gender and BMI (HR=2.50, 95% CI=1.31–4.75, p=0.005), but not of poor PFS. Interestingly, PC3X was independently predictive of poor PFS and OS when it was adjusted for AFP (PFS: HR=1.74, 95% CI=1.01–2.98, p=0.045 and OS: HR=2.21, 95% CI=1.15–4.27, p=0.018). In addition, when high PC3X and high AFP were combined, they were independently predictive of poor PFS and OS when adjusted for Child-Pugh score (PFS: HR=2.68, 95% CI=1.36–5.28, p=0.004 and OS: HR=8.17, 95% CI=3.31–20.13, p<0.0001), age, gender and BMI (PFS: HR=2.81, 95% CI=1.38–5.74, p=0.004 and OS: HR=6.35, 95% CI=2.66–15.15, p<0.0001), and when adjusted for size of largest lesion, number of lesions and presence of portal vein invasion (PFS: HR=2.01, 95% CI=0.99–4.07, p=0.053 and OS: HR=4.20, 95% CI=1.66–10.62, p=0.003), though only borderline statistically significant for PFS (Table 3).

Discussion

We investigated a biomarker reflecting increased type III collagen deposition and cross-linking (PC3X) and evaluated its potential in HCC in comparison to PRO-C3 and AFP. The main findings were that: 1) PC3X was significantly elevated in plasma from patients with HCC compared to those with non-cirrhotic HBV infection, cirrhosis, and healthy controls, 2) high levels of PC3X in HCC patients were associated with shorter PFS and OS, 3) PC3X as a prognostic biomarker was superior to PRO-C3 and independent of AFP, and 4) PC3X and AFP when combined had additive prognostic value versus either alone. Thus, this is the first study to show that cross-linked type III collagen pro-peptides, a marker of the ECM has biomarker potential in primary liver cancer.

Fibrosis-associated myofibroblasts, collagen deposition and liver stiffness are key features of the hepatic premalignant environment. At the level of the cellular microenvironment, they play essential roles in HCC development and progression, suggesting that collagens could be an important source of biomarkers.[11,33–35] We found cross-linked multimeric type III collagen pro-peptides (PC3X) in the circulation at higher levels in the cancer patients compared to those with other liver diseases and healthy controls. Similar findings were observed for the well-known liver fibrosis biomarker PRO-C3. However, whereas a significant difference in PRO-C3 was observed between controls and patients with non-cirrhotic HBV infection, no difference was seen in PC3X levels. In addition, when evaluating the correlation of PRO-C3 and PC3X in healthy and pathological conditions, the two markers were not coordinately altered. Whereas the correlation was pronounced in patients with cirrhosis and HCC, this was not the case in healthy controls and non-cirrhotic HBV infected patients. This suggests that late-stage fibrosis states (cirrhosis and HCC) are characterized by a more homogeneous composition of the ECM, and with regard to type III collagens, are more cross-linked. It also implies that as a biomarker for HCC, PC3X is likely to have superior performance to PRO-C3.

Cross-linking and matrix stiffness correlate with HCC risk and tumor progression.[12,36] Furthermore, the enzymes LOXL2 and TG2 responsible for cross-linking have been shown to have prominent roles in HCC invasion and metastasis.[15–17] Consistently, we found that high PC3X levels were predictive of poor PFS and OS, whereas high levels of PRO-C3 were not. This suggests that PC3X has additional potential as a prognostic biomarker of cancer outcomes, and is superior to PRO-C3.

We found that high PC3X was predictive of poor PFS and OS when adjusted for age, gender and BMI, covariates that can affect collagen turnover.[37] High PC3X was however not predictive for PFS when adjusted for Child-Pugh score. This is not surprising as the Child-Pugh score is based on liver function parameters that are affected by the severity of fibrosis.[38] We also found that PC3X was not predictive for PFS or OS when adjusted for size of largest lesion, number of lesions and presence of portal vein invasion. Again, LOXL2-mediated collagen cross-linking has been shown to act as invasion highways promoting both intrahepatic and extrahepatic metastasis implying that the degree of cross-linking of collagens associates with the number of lesions and with advanced disease.[15]

Similar to other studies, we detected high AFP levels in HCC patients and high levels were associated with poor outcomes.[39,40] However, 58% of cancer patients had AFP levels (<20 IU/mL) that would not usually prompt further investigation; the large difference between cirrhosis and HCC was driven by a subgroup with very high AFP levels secreted by the malignant hepatocytes. Since AFP secretion can vary widely between malignant hepatocytes, as a biomarker it has low sensitivity, a major limitation to its diagnostic use. Interestingly, and as would be expected, PC3X as a matrix-derived biomarker had diagnostic value in the subgroup of HCC patients with low/normal AFP levels. Thus, though PC3X may not have superior diagnostic performance compared to AFP, PC3X does provide additional diagnostic value suggesting potential as a risk assessment marker. Moreover, high PC3X was independently predictive of poor PFS and OS when adjusted for AFP supporting the argument that PC3X measures a pathologically distinct aspect of tumor biology as compared to tumor-derived AFP (see Figure 1 in Supplemental Digital Content 5). In addition, we detected improved prognostic potential when combining PC3X and AFP emphasizing the importance of assessing ECM as well as tumor-cell properties in HCC.

In sum, PC3X is a novel marker of ECM deposition and stiffness that is associated with HCC outcomes. We speculate that cross-linked type III collagen pro-peptides are released to the circulation from the hepatic premalignant environment in cirrhotic patients, and in increased levels from the tumor microenvironment. This is likely associated with the high activity of cancer-associated fibroblasts (CAFs) and cross-linking enzymes (see Figure 1 in Supplemental Digital Content 5).[11,41] LOXL2 and TG2 are increasingly considered therapeutic targets to reduce matrix stiffness and attenuate cancer progression. Thus, PC3X may also have predictive potential in this setting.[42,43]

The limitations of this study include that it is a relatively small HCC cohort that comprises patients with both early- and late-stage disease, who were treated with different treatment regimens, thus impacting differentially on outcomes. Thus, larger studies are required to validate the biomarker potential of PC3X in HCC.

Conclusion

In conclusion, cross-linked multimeric type III collagen pro-peptides can be used as a biomarker of tissue stiffness in HCC and as we show, they measure a pathophysiologically distinct aspect of tumor biology than AFP. PC3X was higher in HCC compared to other liver diseases and healthy controls. In addition, high levels of PC3X in cancer were associated with shorter PFS and OS suggesting that this marker has prognostic potential.

Abbreviations

AFP, alpha-fetoprotein; ALT, alanine transaminase; AASLD, American Association for the Study of Liver Diseases; AUROC, area under the receiver operating characteristics; APASL, Asian Pacific Association for the Study of the Liver; AST, aspartate transaminase; BCLC, Barcelona Clinic Liver Cancer; BMI, body mass index; CAFs, cancer-associated fibroblasts; PC3X, cross-linked pro-peptides of type III collagen; EASL, European Association for the Study of the Liver; ECM, extracellular matrix; HR, hazard ratios; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HRP, horseradish peroxidase; KM, Kaplan–Meier; LOX, lysyl oxidase; LOXL, LOX-like; OS, overall survival; PLT, platelet count; PFS, progression-free survival; SD, standard deviation; TMB, tetramethylbenzidine; TACE, transarterial chemoembolization; TG2, transglutaminase 2.

Data Sharing Statement

The data obtained in the current study are included in this published article, available from the corresponding author or available from the indicated sources.

Ethics Approval and Informed Consent

The study was approved by the Human Ethics Committee of the Sydney West Area Health Service (HREC No.2002/12/4.9 (1564)) in compliance with the Helsinki Declaration. Written informed consent was obtained from all participants.

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Disclosure

C. Jensen, S. Holm Nielsen, F. Genovese, M. J. Nielsen, M. A. Karsdal, D. J. Leeming and N. Willumsen are employed at Nordic Bioscience which is a company involved in discovery and development of biochemical biomarkers. M. A. Karsdal, F. Genovese and D. J. Leeming own stocks in Nordic Bioscience. The authors report no other conflicts of interest in this work.

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