Supplemental Digital Content 1

MATERIAL AND METHODS

Reagents

All reagents used for the experiments were standard chemicals from Merck (Whitehouse Station, NJ, USA) or Sigma Aldrich (St. Louis, MO, USA), unless otherwise stated. The synthetic immunogenic peptide Ovalbumin (OVA)-CGG-CPTGPQNYSP used for monoclonal antibody production was purchased from Chinese Peptide Company (Beijing, China).

Generation of monoclonal antibodies

The N-proteinase generated neo-epitope of the N-terminal pro-peptide of type III collagen was selected as target and the amino acid sequence 144'-CPTGPQNYSP-'153 in the α1 chain was used to generate an antibody specific for the pro-peptide. The sequence was aligned for homology to rat and mouse and blasted for uniqueness among other human proteins using the NPS@: network protein sequence analysis with the Uniprot/Swiss-Prot database.[1] The sequence CPTGPQNYSP was found unique for the human N-terminal pro-peptide of type III collagen.
Generation of monoclonal antibodies, clone characterization and antibody characterization were carried out as previously described.[2] The monoclonal antibody was found specific for the target sequence CPTGPQNYSP and not to an elongated (CPTGPQNYSPQ) or non-sense peptide (GSPGKDGVRG).[2]
Supernatant from antibody producing hybridoma cells was collected and the monoclonal antibody was purified using HiTrap protein-G-columns (GE healthcare Life Sciences, Little Chalfont, Buckinghamshire, UK) and labeled with the Lightning-Link™ Rapid Biotin Conjugation Kit (Type B) (Innova Biosciences) or Lightning-Link™ HRP Conjugation Kit (Innova Biosciences) according to the manufacturer's instructions.

PC3X ELISA protocol

The PC3X sandwich ELISA was based on use of the monoclonal antibody as both catcher and detector. Consequently, only cross-linked molecules of type III collagen pro-peptide can be recognized by the assay. The PC3X sandwich ELISA procedure was as follows: 96-well streptavidin-coated microtiter plates (Roche, cat. 11940279) were coated with 100 µl of 1 µg/ml biotin-labeled catcher antibody dissolved in assay buffer 1 (50mM phosphate buffered saline with bovine serum albumin (1% w/v), Tween-20 (0.1% w/v), and bronidox (0.36% v/v) (PBS-BTB), 8g/L Sodium Chloride (NaCl), pH 7.4) and incubated for 30 minutes at 20˚C. A volume of 20 µl of standard, controls or sample (diluted 1:2 in assay buffer 2 (50mM PBS-BTB, 8g/L NaCl, pH 7.4 with 5% LiqII)) was added followed by immediate addition of 100 µl of assay buffer 2 and incubated for 20 hours at 4˚C. A standard curve was produced by using supernatant from the "Scar-in-a-jar" (SiaJ) model in which healthy primary human lung fibroblasts were cultured in the presence of ficoll and transforming growth factor-beta (TGF-β) as previously described.[3] One control was produced by using supernatant from the lung fibroblasts and the other control included healthy human serum. Then, 100 µl of 1 µg/ml horseradish peroxidase (HRP)-labeled detector antibody diluted in assay buffer 1 was added and incubated for 1 hour at 20˚C. Next, 100 µl Tetramethylbenzidine (TMB, Kem-En-Tec Diagnostics, Taastrup, Denmark) was added and incubated for 15 minutes at 20˚C in the dark. All incubations included shaking of the plates (300 rpm) followed by five times washing (20mM TRIS, 50mM NaCl, pH 7.2). To stop the reaction of TMB, 100 µl of 1% sulfuric acid was added and the plates were analyzed in a VersaMax ELISA microplate reader at 450 nm with 650 nm as reference. A standard curve was plotted using a quadratic curve fit and data were analyzed using the Softmax Pro v. 6.3 software.

Technical evaluation of the PC3X assay

A two-fold dilution of healthy human serum (n=3) and EDTA plasma (n=3) was used to determine linearity and calculated as percentage recovery of the undiluted sample. Accuracy was measured in healthy human serum samples (one low and one high) and in one sample of supernatant from cultured lung fibroblasts (SiaJ model) spiked with two-fold dilutions of the standard curve and calculated as percentage recovery of the expected concentration (serum or supernatant and standard curve combined). The analyte stability was determined in healthy human serum samples (n=3) exposed for up to four freeze and thaw cycles and calculated as percentage recovery of the first cycle. The analyte stability was further tested by incubating healthy human serum samples for 24 hours or 48 hours at either 4˚C or 20˚C and tested against a sample stored at -20˚C. Interference was determined in healthy human serum spiked with hemoglobin (low=0.078 mM, high=0.155 mM), biotin (low=15 ng/ml, high=45 ng/ml) or lipid (low=2.42 mM, high=5.49 mM) and calculated as the percentage recovery of analyte in non-spiked serum. Furthermore, the interfering effect of human anti-mouse antibody (HAMA) was evaluated. Four healthy human plasma samples were added to a panel of different HAMA concentrations. These were analyzed with and without 5% Liquid II (Osteocalcin EIA Puf-Liq by Roche Diagnostics) in the dilution buffer, which counteracts the interference by HAMA. Lower limit of detection (LLOD) was calculated as the mean + 3xStandard Deviation (SD) of the blank from 21 determinations of standard K (i.e. buffer). Upper limit of detection (ULOD) was determined as the mean – 3xSD of 10 measurements of Standard A. The intra-and inter-assay variation were determined by ten independent runs of seven samples that covered the detection range (LLOD-ULOD). The seven samples included five healthy human serum samples and the two assay controls. The intra-assay variation was determined as the mean coefficient of variance (CV%) within plates, and the inter-assay variation was calculated as the mean CV% between plates. Correlation between PC3X levels in serum and EDTA plasma was determined in matched serum and EDTA plasma samples from 17 healthy human donors.

REFERENCES

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3. Chen ZCC, Peng Y, Raghunath M. The Scar-in-a-Jar: Studying Antifibrotic Lead Compounds from the Epigenetic to the Extracellular Level in One Well. *IFMBE Proc*. 2009;23:1499-1502. doi:10.1007/978-3-540-92841-6_371

Supplemental Digital Content 2

RESULTS

Technical evaluation of the PC3X assay

A technical validation was performed to evaluate the PC3X assay. A summary of the data is shown in Table 1. The detection range (LLOD-ULOD) of the assay was 0.4 to 17.3 ng/ml. The intra- and inter-assay variation was 8% and 12%, respectively, which were below the acceptance levels on 10% and 15%. Linearity was observed from undiluted to a four-fold dilution for human serum and EDTA plasma with a 107% and 117% dilution recovery, respectively. Spiking the calibrator peptide into human serum or into supernatant from cultured activated lung fibroblasts resulted in a mean recovery of 108% and 100%, respectively. The analyte was recoverable for four freeze-thaw cycles (99%) and after prolonged storage of samples at 4˚C for 24 or 48 hours (119% and 104%) and at 20˚C for 24 or 48 hours (103% and 104%). Biotin, lipids, hemoglobin and HAMA did not interfere with PC3X measurements, with recoveries ranging from 81% to 102%. All analyte recoveries were accepted as they were within 100±20%. A high correlation between PC3X values in human serum and EDTA plasma was detected (r=0.95, p<0.0001), demonstrating that levels in serum and EDTA plasma are comparable and that both biological matrices can be utilized.

Table 1. Technical validation of the PC3X assay

Technical validation step	Results
Detection range (LLOD-ULOD)	0.4 ng/ml-17.3 ng/ml
Intra-assay variation	8 %
Inter-assay variation	12 %
Dilution recovery of serum	107 %
Dilution recovery of EDTA plasma	117 %
Spiking recovery in serum	108 %
Spiking recovery in lung fibroblast supernatant	100 %
Freeze-thaw recovery of serum	99 %
Analyte stability of serum 24h, 4˚C/20˚C	119/103 %
Analyte stability of serum 48h, 4˚C/20˚C	104/104 %
Biotin recovery of serum, low/high	98/91 %
Lipemia recovery of serum, low/high	102/94 %
Hemoglobin recovery of serum, low/high	102/98 %
HAMA recovery of EDTA plasma	81 %
Correlation for EDTA plasma and serum	r=0.95, p<0.0001

Percentages are reported as mean. Pearson's correlation coefficient was used to evaluate the correlation of PC3X in EDTA plasma and serum. Abbreviations: LLOD, Lower limit of detection; ULOD, Upper limit of detection.

Supplemental Digital Content 3

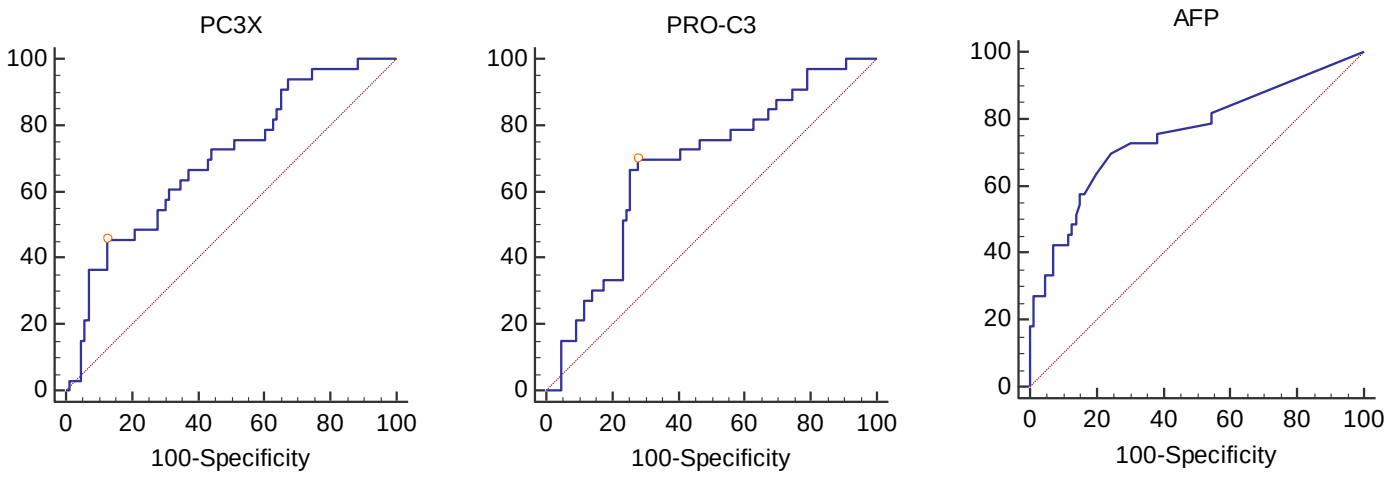

Figure 1: Receiver operating characteristics analysis evaluating the ability of PC3X, PRO-C3 and AFP to separate patients with early stage HCC (BCLC A/0) from patients with cirrhosis.

Supplemental Digital Content 4

Table 1. Correlation table

		AFP	AGE	Albumin	ALT	AST	Bilirubin	PLT	PC3X	PRO-C3
AFP	r		-0.07	-0.20	**0.29**	**0.29**	0.03	-0.13	**0.25**	**0.30**
	p		0.541	0.083	**0.011**	**0.009**	0.789	0.242	**0.026**	**0.007**
AGE	r	-0.07		-0.01	-0.13	**-0.25**	-0.10	-0.03	**-0.41**	**-0.35**
	p	0.541		0.957	0.257	**0.026**	0.378	0.820	**0.0001**	**0.002**
Albumin	r	-0.20	-0.01		-0.10	**-0.33**	**-0.43**	**0.43**	-0.18	**-0.26**
	p	0.083	0.957		0.370	**0.003**	**0.0001**	**0.0001**	0.124	**0.022**
ALT	r	**0.29**	-0.13	-0.10		**0.82**	0.19	-0.17	**0.37**	**0.38**
	p	**0.011**	0.257	0.370		**<0.0001**	0.099	0.145	**0.0009**	**0.0006**
AST	r	**0.29**	**-0.25**	**-0.33**	**0.82**		**0.39**	-0.15	**0.38**	**0.48**
	p	**0.009**	**0.026**	**0.003**	**<0.0001**		**0.0004**	0.194	**0.0006**	**<0.0001**
Bilirubin	r	0.03	-0.10	**-0.43**	0.19	**0.39**		**-0.42**	0.11	0.18
	p	0.789	0.378	**0.0001**	0.099	**0.0004**		**0.0001**	0.321	0.116
PLT	r	-0.13	-0.03	**0.43**	-0.17	-0.15	**-0.42**		-0.03	-0.07
	p	0.242	0.820	**0.0001**	0.145	0.194	**0.0001**		0.783	0.547
PC3X	r	**0.25**	**-0.41**	-0.18	**0.37**	**0.38**	0.11	-0.03		**0.72**
	p	**0.026**	**0.0001**	0.124	**0.0009**	**0.0006**	0.321	0.783		**<0.0001**
PRO-C3	r	**0.30**	**-0.35**	**-0.26**	**0.38**	**0.48**	0.18	-0.07	**0.72**	
	p	**0.007**	**0.002**	**0.022**	**0.0006**	**<0.0001**	0.116	0.547	**<0.0001**	

Abbreviations: AFP, alpha-fetoprotein; ALT, alanine transaminase; AST, aspartate transaminase; PLT, platelet count; r, spearman rank correlation coefficient.

Table 2. Multiple regression analysis predicting PC3X, PRO-C3 and AFP

	PC3X (p-value)	PRO-C3 (p-value)	AFP (p-value)
Child-Pugh score	0.346	0.887	0.321
Size of largest lesion	0.554	0.825	0.325
Number of lesions	**0.001**	0.577	0.763
Metastasis Y/N	0.905	0.404	0.644
Portal vein invasion Y/N	0.240	0.981	**0.019**

Abbreviations: BCLC, Barcelona clinic liver cancer; Y, yes; N, no.

Supplemental Digital Content 5

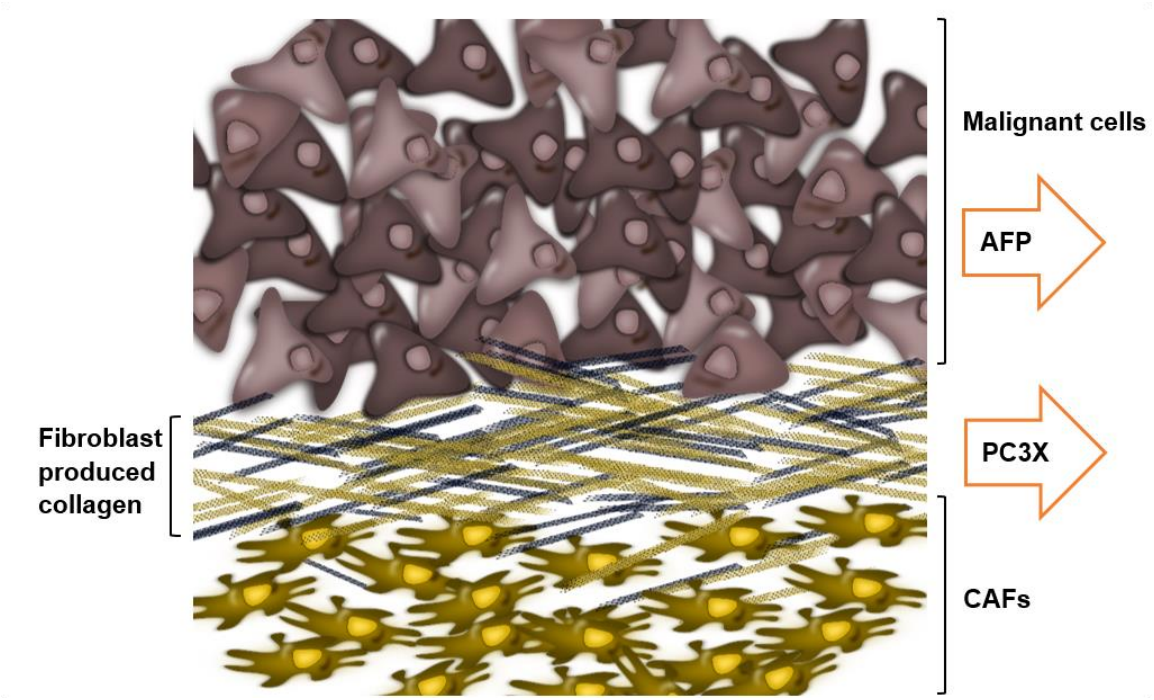

Figure 1: PC3X and AFP reflect two different aspect of tumor biology. Whereas PC3X reflects fibroblast and cancer associated fibroblast (CAF) produced type III collagen and cross-linking in the tumor microenvironment, AFP is derived from the malignant cells.