

Peptides in low molecular weight fraction of serum associated with hepatocellular carcinoma

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Abstract. The incidence of hepatocellular carcinoma (HCC) in the United States is increasing and the increase is projected to continue for several decades. The overall survival of HCC patients is poor and treatments are not effective in part because most of the diagnoses come at a late stage. The development of new markers for detection of HCC would significantly improve patient prognosis. This paper describes identification of candidate markers previously reported in our serologic study of an Egyptian population by quantitative comparison of matrix assisted laser desorption ionization time of flight (MALDI-TOF) mass spectra. To identify these marker candidates, we performed LC-MS/MS sequencing that identified nine native peptides associated with HCC, including two reported previously. Four truncations of N terminus of complement C3f and a fibrinopeptide increased in control sera; two complement C4 α peptides, a zyxin peptide, and a coagulation factor XIII peptide increased in cancer patient sera. We have also identified increased biliverdin diglucuronide in the sera of cancer patients. These peptides could potentially serve as markers of HCC following additional validation studies; however, association of similar peptides with other diseases and cancers dictates a very cautious approach.

Keywords: Hepatocellular carcinoma, mass spectrometry, serum, complement, biliverdin diglucuronide

1. Introduction

Hepatocellular carcinoma (HCC) is a highly fatal cancer that affects annually approximately half a million people worldwide [1,2]. Liver cancer ranks fifth in frequency of cancers in the world. Since the mid-1980s, the incidence of HCC in the United States has been rising and this increase is predicted to continue in the next few decades primarily due to hepatitis C viral (HCV) infection [2–5]. The overall survival rate of HCC patients is poor which is in part related to the lack of reliable tools for early diagnosis. A diagnosis of HCC is often made at a late stage when the disease is too advanced for an effective treatment. Surveillance of patients at high risk for developing HCC is

an important strategy that can potentially decrease the cancer-related mortality.

Chronic hepatitis C viral infection with subsequent liver cirrhosis is the major risk factor for development of HCC [2,6,7]. Patients with cirrhosis are recommended to undergo routine surveillance. Diagnosis is most often based on radiological imaging. Abdominal ultrasound is the most common imaging modality used in the surveillance of HCC, but it is operator dependent and limited in its ability to differentiate HCC from such non-neoplastic lesions as regenerative nodules [2,8]. The only serologic marker for HCC surveillance is alpha fetoprotein (AFP). AFP has reported sensitivity of 39 to 65%, specificity of 76 to 94%, and positive predictive values of 9% to 50% [2,9]. This is not sufficient for an efficient detection of HCC. New biomarkers for early detection and prevention of HCC are needed and a search is under way in several laboratories [10–13]. The management of the disease is predicted to benefit from identification of appropriate serologic markers

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that would assist with the optimization of therapeutic decisions.

It was shown that the presence of tumors is associated with changes in the composition of native peptides in serum [14]. Liver is a major source of circulating proteins and HCC is expected to induce significant perturbations. Proteases and peptidases are reportedly deregulated in HCC [15–18] and it is reasonable to expect that cancer-specific peptides and fragments of proteins will be found in the low molecular weight fraction of serum [19–21].

Our laboratory has developed a denaturing ultrafiltration method for enrichment of the LMW serum proteins and peptides for MALDI-TOF mass spectrometric analysis [19,20]. The method has allowed a fast comparison of hundreds of patient samples which is expected to decrease the gap between marker discovery and clinical validation [22]. In previous articles, we reported consistent differences associated with HCC in a large sample of patients from an Egyptian population (73 HCC cases, 72 cancer-free controls, and 52 chronic liver disease controls) [19,21]. Here we describe mass spectrometric identification of nine of the differentially abundant peptide peaks which will further facilitate examination of their association with HCC.

2. Experimental procedures

2.1. Materials

AmiconUltra-4 Centrifugal Filter Devices with 30 kDa cut-off were purchased from Millipore (Bedford, MA, USA). Sep-Pak Vac RC C18 Cartridges were bought from Waters Corporation (Milford, MA). BCA protein assay was purchased from Pierce Biotechnology (Rockford, IL, USA). α -cyano-4-hydroxycinnamic acid (CHCA) was purchased from Bruker Daltonics (Billerica, MA, USA). Opti-TOF 384 well MALDI plate and mass calibration standards were purchased from Applied Biosystems (Framingham, MA). Other chemicals and solvents were purchased from Sigma-Aldrich (St. Louis, MO, USA); solvents were of HPLC grade.

3. Blood sample collection and preparation

Serum samples of HCC cases and controls with(out) chronic liver disease were obtained in collaboration with the National Cancer Institute of Cairo University,

Egypt from 2000 to 2002 as described previously [19, 21]. Blood samples were collected by a trained phlebotomist around 10 am and processed within a few hours according to a standard protocol. Aliquots of sera were frozen at -80°C immediately after processing until analysis; all mass spectrometric measurements were performed on twice-thawed sera. For sequencing of peptides, 0.5 ml of serum was diluted in 2.5 ml 8M guanidine hydrochloride and ultrafiltered at 3,000 x g for 90 minutes at 4°C using 30-kDa AmiconUltra membrane (Millipore). The ultrafiltrate was desalted on a Sep-Pak C18 cartridge (Waters) according to the manufacture's protocol and dried in a centrifugal vacuum evaporator.

4. Fractionation of proteins

The dried ultrafiltrate was resuspended in 20 μL 0.1% TFA/water and separated on Chromolith Performance RP-18 Column ($100 \times 4.6 \text{ mm}$) (EMD Chemicals, NJ) at room temperature. A flow (1 mL/min) of solvent gradient (5% of solvent B for 2 min, 5–40% of solvent B for 23 min, 40–95% of solvent B for 2 min, 95% of solvent B in 3 min, 95–5% solvent B in 1 min, and 5% of solvent B for 10 min; Solvent A: 100% water / 0.1% TFA, Solvent B: 100% acetonitrile / 0.1% TFA) was used for separation using an Agilent 1100 HPLC system with fractions collected every 30 seconds from 0 to 30 minutes. The fractions were dried in a centrifugal vacuum evaporator. Each fraction was analyzed using MALDI-TOF or ESI-QqTOF mass spectrometers as described below.

5. MALDI-TOF/TOF analysis of peptides

A 4800 MALDI TOF/TOF TM Analyzer (Applied Biosystems, CA) was used for the peptide analysis. Each dried fraction was resuspended in 1 μL matrix (α -Cyano-4-hydroxycinnamic acid, 3.3 mg/mL in 50% Acetonitrile/ 50% water/ 0.1% TFA) and spotted on a MALDI plate for analysis in positive ion mode in 800–5,500 m/z range. In both reflector and MS/MS mode, laser repetition rate was 200 Hz and the instrument default calibration was used. Precursor list for MS/MS was generated for all fractions and the top 15 ions were selected for MS/MS analysis. The ions of interest as the biomarker candidates were selected for MS/MS analysis if they were not in the precursor list. In MSMS mode, 2 kV collision energy (with CID gas on) was used to fragment the peptides.

Table 1
Sequences of identified peptides and a metabolite associated with HCC [19,21]

Monoisotopic mass	Sequence	Protein source	Increased in	MASCOT score	Expect
1863.98	SSKITHRIHWESASLL	Complement C3	Control	76	0.0023
1776.95	SKITHRIHWESASLL	Complement C3	Control	83	0.00027
1689.92	KITHRIHWESASLL	Complement C3	Control	76	0.0012
1561.83	ITHRIHWESASLL	Complement C3	Control	78	0.00072
1738.91	NGFKSHALQLNNRQI	Complement C4A	HCC	115	3.2e-7
2377.19	DDPDAPLQPVTPLQLFEGRRN	Complement C4A	HCC	106	4e-6
2530.26	VPPNNSNAEEDDLPTVELQGVVPR	Coagulation factor XIII A	HCC	135	5.1e-9
1379.57	ADSGEGDFLAEGGGV	Fibrinogen alpha	Control	63	0.0086
2303.20	HVQPQPQPKPQVQLHVQSQT	Zyxin	HCC	122	3e-8
933.31	C ₄₅ H ₅₀ N ₄ O ₁₈	Biliverdin diglucuronide	HCC	N/A	N/A

6. NanoLC-QqTOF analysis of peptides

Each dried fraction was redissolved in 5 μ L 0.1% formic acid/water and separated on a Nano Acquity Ultra Performance LC system (Waters). The sample was desalted on a Symmetry C18 180 μ m i.d. \times 20 mm pre-column (Waters) and then injected onto a BEH130 C18 100 μ m i.d. \times 100 mm analytical column (Waters). The peptides were eluted with a gradient of 5–20% of solvent B for 5 min, 20–40% of solvent B for 30 min, 95% of solvent B in 5 min, 95–5% solvent B in 1 min, and 5% of solvent B for 18 min; Solvent A: 100% water / 0.1% formic acid, Solvent B: 100% acetonitrile / 0.1% formic acid. The flow rate was 300 nL/min and the effluent was introduced into the QSTAR Elite ESI-QqTOF mass spectrometer (Applied Biosystems, Foster City, CA) via an uncoated 15 μ m i.d. PicoTip Emitter (New Objective Inc., Woburn, MA). The spray voltage was 2300 V. The mass spectrometer was set to do the Information Dependent Acquisition (IDA) which automatically acquire TOF MS (survey) spectra at the m/z range 350–2500 Da and MS/MS (dependent) spectra on the five most intense doubly or triply charged peptides. The precursor ions selected previously were excluded for 60 s and an inclusion list of biomarker candidates was used.

7. Peptide identification and sequencing

The database searches for peptide identification were performed using Mascot and PepArML. For Mascot analysis, the raw MS/MS spectra were processed using Mascot Distiller and searched against the NCBI human database. No enzyme was considered in these searches. MS peptide tolerance was 100 ppm and MS/MS tolerance was 0.3 Da. Peptides identified with E-values < 0.01 are shown in Table 1. For peptide identification using PepArML, MS/MS spectra were

reformatted as mzXML format using PyMsXML (HU http://www.bioinformatics.org/groups/?group_id=701). The resulting files were searched against the IPI-Human protein sequence database using the PepArML meta-search engine, incorporating search engines Mascot, X!Tandem, OMSSA, and Myrimatch. Search engines were configured for non-specific peptides, precursor tolerance 2.0 Da, fragment tolerance 0.4 Da, fixed carbamidomethyl Cys modification, and variable Met oxidation, pyro-Glu on N-terminal Glu and Gln, and pyro-carbamidomethyl on N-terminal Cys modifications. In addition to searches against the target IPI-Human protein sequence database, searches against ten independently shuffled decoy sequence databases were carried out using otherwise identical search parameters. The PepArML result combiner was used to unify the target and decoy results from the four search engines. The results of one decoy search was used for internal PepArML calibration, while the results of the other nine decoy searches were used to estimate the false discovery rate (FDR) of PepArML's peptide identifications. The unsupervised PepArML machine-learning algorithm used each search engine's E-value, the precursor mass delta, and the number of agreeing search engines to distinguish correct from incorrect peptide identifications. PepArML peptide identifications with estimated FDR of 10% or better were considered in the analysis.

8. Results

8.1. Identification of candidate biomarkers for the detection of hepatocellular carcinoma

We have previously reported mass spectrometric quantification that identified differentially abundant peptides associated with HCC; these studies compared 73 HCC cases, 72 age- and gender- matched cancer-free

controls, and 52 controls with chronic liver disease [19–21]. The studies focused on native (non-tryptic) peptides in the low mass fraction of serum enriched by denaturing ultrafiltration. In those studies, the sequencing by direct MALDI-TOF/TOF fragmentation was successful for only a limited number of peptides. Here we report LC-MS/MS identification of the differentially abundant peptides that separated HCC patients from the healthy and chronic liver disease controls (Table 1) [19–21].

To identify the marker candidates, we enriched native peptides/low mass proteins by denaturing ultrafiltration in 8M guanidine HCl followed by reverse phase fractionation on a monolithic HPLC column. We have analyzed samples from cancer patients, chronic liver disease patients, and disease free controls and found the fractionation reproducible with candidate peptides eluting in the same fractions across the samples (data not shown). Each fraction was first analyzed by MALDI-TOF/TOF which provided a link to the MALDI-TOF quantitative analyses. Fragmentation of the enriched peptides provided sufficiently rich b and y ion series to allow high confidence identification of complement fragments and other non-tryptic peptide candidates (FDR < 0.05). We used nanoLC-MS/MS to confirm identification of the peptides and to identify additional peptides that did not provide sufficient fragment information using the MALDI ionization.

The peptide at m/z 1865, consistently higher in healthy and chronic liver disease controls in our previous studies [19–21], has been identified previously as the fragment of complement C3f with the sequence SSKITHRIHWESASLL. In this study, we identified consistently five additional peptide fragments of C3f with the same C terminus. Three of these have also higher observed intensities in our previous comparison of chronic liver disease controls compared to HCC. Two peptides (m/z 1739, 2377), elevated in sera of HCC patients, were identified as fragments of complement C4a (Table 2). We list all the identified peptides from the complement proteins, including the candidate markers, in Table 2. The ladder-like pattern (sequential one amino acid shortening at the N- and/or C-termini) indicates that exoproteases are likely involved in the processing of the peptides, as described previously [23].

The candidate marker at m/z 2,530, which is increased in HCC patients, was identified as a 24-amino acid peptide from coagulation factor XIII A with an arginine at the C terminus (Table 1). The peptide identity was confirmed by complementary fragmentation of the peptide in several different patient samples which

further increases confidence of the identification. The complementary fragments from spectra of the same peptide obtained from multiple samples were observed for a number of the peptides in our study (data not shown). Two additional sequenced peptides were associated with HCC (Table 1). A peptide at m/z 2,303 was identified as a zyxin peptide using LC-MALDI-MS/MS and a peptide at m/z 1,379 was sequenced as a fibrinogen α peptide using nanoESI-MS/MS.

One of the peaks at m/z 933, increased in sera of HCC patients [19], is not a peptide. The MS/MS spectra of this compound showed a dominant fragment at m/z 759 which corresponds to the loss of glucuronide (Fig. 1). Analysis of the smaller fragments at m/z 741, 583, 473 and 298 is consistent with the structure of biliverdin diglucuronide which confirms the unexpected finding of a metabolite in our study of peptidic marker candidates.

9. Global peptide identification (from LMW human blood fraction using LC-MS/MS)

Human serum is a complex mixture containing more than 10^4 proteins with wide range of physico-chemical properties and an immense dynamic range ($\sim 10^8$) [24]. To improve our sequencing effort, we employed denaturing ultrafiltration to enrich the LMW fraction of serum or plasma; native peptides were further separated by reverse phase chromatography. The sequencing in the serum samples identified a total of 245 native peptides deriving from 48 proteins (Table 3). We observed ladder-like truncations at either N- or C-termini of the identified sequences as reported by Villanueva and colleagues [14,24]. About 20% of the sequences belong to fibrinogen alpha, the dominant component of the LMW fraction of serum. We analyzed samples of HCC patients with high ion intensity of those candidates in order to identify some of the peptides with low intensity in controls. We found that 27 peptides that were identifiable only in patient sera including peptides with m/z 2,530 and 2,303 (Table 1). In addition to serum, we also examined plasma and found that at least twenty peptides deriving from fibrinogen α , inter-alpha-trypsin inhibitor heavy chain H4, thymosin beta-4, zyxin, alpha-2-HS-glycoprotein precursor, fibrinogen beta, complement C4 and serum albumin were also present in plasma.

Table 2
Peptide ladders derived from complement. Bolded complement C3 peptides are higher in controls; bolded complement C4 peptides are higher in HCC; regular font peptides did not change [19,21]

Protein name	Observed mass	Sequence
Complement C3	2021.10	SSKITHRIHWESASLLR
	1934.07	SKITHRIHWESASLLR
	1864.99	SSKITHRIHWESASLL
	1777.97	SKITHRIHWESASLL
	1690.94	KITHRIHWESASLL
	1562.84	ITHRIHWESASLL
	1211.65	RIHWESASLL
	942.47	HWESASLL
	1855.85	SEETKENEFTVTAEGK
	1768.82	EETKENEFTVTAEGK
	1471.74	AAVYHHFISDGV
	1206.61	SVQLTEKRMD
	1816.89	SNLDEDIIAEENIVSR
	2089.05	GYTQLAFRQPSSAFAAFV
	Complement C4A	1626.84
1739.92		NGFKSHALQLNNRQI
1896.028		NGFKSHALQLNNRQIR
1781.99		GFKSHALQLNNRQIR
1625.88		GFKSHALQLNNRQI
1512.80		GFKSHALQLNNRQ
1449.81		SHALQLNNRQIR
1293.70		SHALQLNNRQI
1052.56		SHALQLNNR
2378.19		DDPDAPLQPVTPLQLFEGRRN
2108.07		DDPDAPLQPVTPLQLFEGR
2225.12	ALEILQEEDLIDEDDIPVR	

10. Discussion

Incidence of hepatocellular carcinoma (HCC) in the US increases and has been associated with hepatitis C (HCV) viral infection. Very high rates of HCC are observed in Egypt where HCV presents a serious health problem [25,26]. Our studies of native peptides in serum samples from the Egyptian population identified several peptides associated with the development of HCC [19–21] that could potentially serve as a serologic marker.

We have reported previously the identification of two peptides m/z 1864 and 1739 [19]. In this continuation of our previous studies, we describe identification of seven new peptides and a metabolite associated with HCC. These peptides derive from complement C3 (m/z , 1777, 1690 and 1562), complement C4 (m/z 2377), coagulation factor XIII (m/z 2530), zyxin (m/z 2303), and fibrinogen α (m/z 1379); the spectra of a metabolite associated with HCC are consistent with biliverdin diglucuronide (m/z 933) (Fig. 1). These proteins are produced by the liver and it is plausible that differential enzymatic processing and distribution associated with liver disease produces peptides diagnostic of the disease

progression. We did observe an association of these peptides with HCC in a study of approximately 200 patients progressing from HCV infection to HCC [19–21]. A combination of the peptides was able to identify HCC with better than 90% prediction accuracy [22,27]. While differential processing of these relatively abundant serum proteins is likely to reflect the progression of liver disease, the specificity for the detection of HCC will have to be carefully evaluated [28]. Indeed, some of the peptides were previously associated with other cancers [29–32] and may be progressively changing with the development of pre-malignant liver disease. In our study, we did observe a strong association of HCC cases and population controls with fibrinopeptide (m/z 1379), coagulation factor XIII A (m/z 2530), and biliverdin diglucuronide (m/z 933) but these molecules did not separate well HCC from cirrhotic controls. The complement C3 and C4 peptides and a peptide derived from zyxin were best at separating HCC patients from cirrhotic controls. The prediction accuracy for the detection of HCC has to be further evaluated by independent methods in samples from an appropriate population before any claims of biomarker potential can be made.

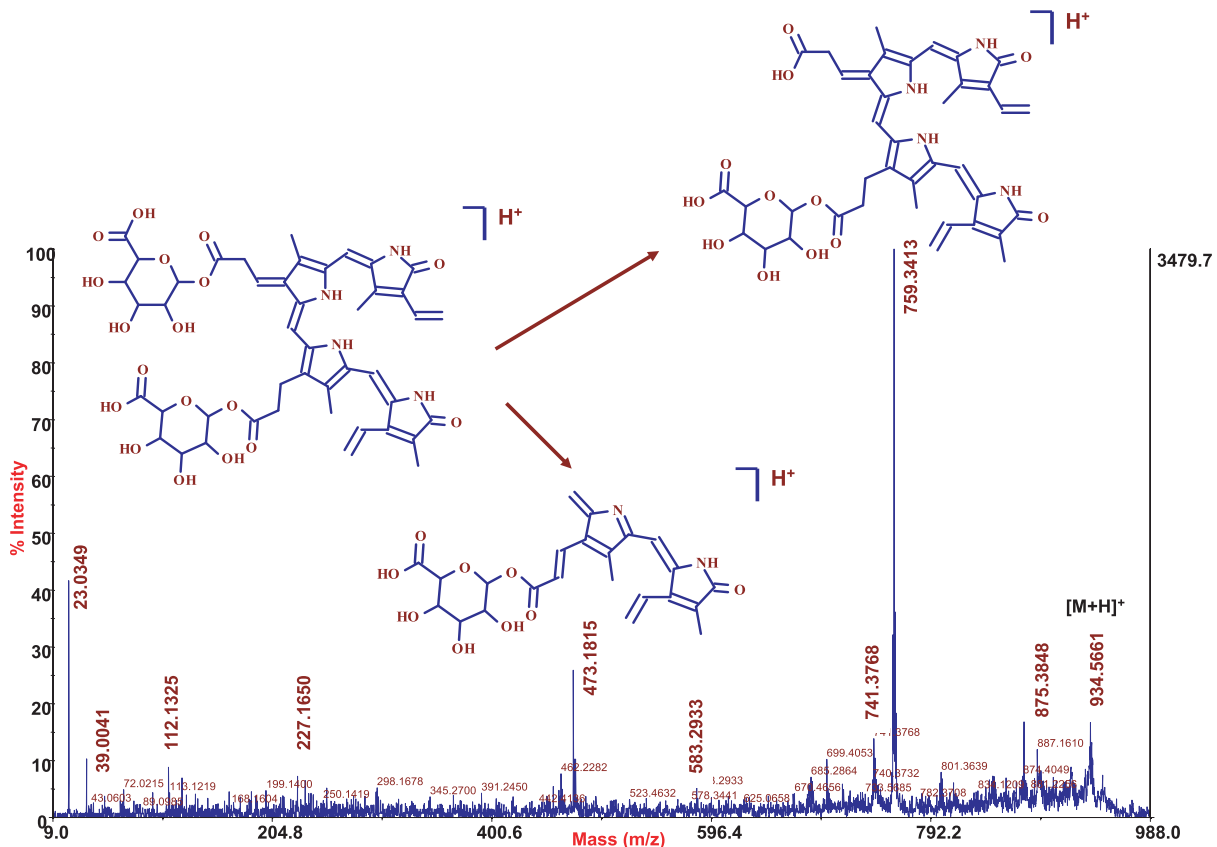


Fig. 1. Tandem mass spectrum of the analyte observed at m/z 934.32 is consistent with the fragmentation of biliverdin digluconide.

Three peptides from complement C3f (m/z 1864, 1777, 1690 and 1562) decrease progressively from disease free controls to chronic liver disease and HCC [19–21]. In contrast, two peptides derived from complement C4 are more abundant in the sera of HCC patients. The changes in complement C3 have been associated with a number of malignancies including T-cell leukemia [30], nasopharyngeal cancer [31], breast cancer [14], and chronic B-cell lymphoid malignancy [33]. Differential expression of complement C4 peptides was also observed in several case-control studies [14,34]. We expected that our denaturing ultrafiltration procedure will enrich the samples compared to the published studies of whole serum [14,34]. We also expected that the disease of the liver, an organ producing majority of the proteins identified in serum, will affect peptide composition more than other cancers. However, we observe in our study a similar set of peptides.

Complement is a mediator of inflammation and contributes to the regulation of immune responses. The complement system contains more than 30 glycoproteins which regulate liver regeneration [35,36], immune

responses [37,38] and other important processes in many as yet incompletely characterized ways [39]. The observed complement peptides are postulated to derive from endoproteolytic cleavage of C3 and C4 proteins followed by exopeptidase trimming [14,34]. Three biochemical pathways are known to activate the complement system: the classical complement pathway, the alternative complement pathway, and the mannose-binding lectin pathway. C3 plays a central role in the activation of the complement system of all three pathways [40]. The complement peptides have different abundance in various diseases [14,19,34,40,41] which indicates that they serve important functions but may decrease their applicability as specific markers. It is interesting to note, however, that a tumor specific exopeptidase processing and activity screening of complement peptides was recently proposed for cancer-specific detection [24].

The peptide m/z 2,303, increased in the serum of HCC patients, derives from zyxin. Upregulation of zyxin in HCC tumors had been previously observed by quantitative reverse transcription-polymerase chain re-

Table 3

Serum proteins whose native peptides were identified in this study. The peptide# indicates numbers of native peptides observed for each of the proteins

Protein name	Access #	Peptide #
Fibrinogen alpha chain	P02671	47
Inter-alpha-trypsin inhibitor heavy chain H4	Q14624	25
Complement C3	P01024	14
Apolipoprotein A-I	P02647	14
Kininogen-1	P01042	13
Zyxin	Q15942	12
Complement component 4A	P0C0L4	12
Fibrinogen beta chain	P02675	12
Apolipoprotein A-IV	P06727	12
Prothrombin	P00734	9
Alpha-2-macroglobulin	P01023	6
Serotransferrin	P02787	6
Plexin domain-containing protein 2	Q6UX71	4
Transferrin	P02766	4
Alpha-2-HS-glycoprotein precursor	P02765	4
Thymosin beta-4	P62328	3
Coagulation factor XIII A chain precursor	P00488	3
Haptoglobin	P00738	3
Alpha-1-antitrypsin	HP01009	3
Clusterin	P10909	3
Serglycin	P10124	2
Serum albumin	P02768	2
Apolipoprotein C-III	P02656	2
Apolipoprotein E	P02649	2
Hemopexin	P02790	2
Serum amyloid-A4 protein	P35542	2
Complement C1r	P00736	2
Vitronectin	P04004	2
Collagen alpha-3(V) chain	P25940	1
Collagen alpha-5(IV) chain	P29400	1
Glycogen debranching enzyme	P35573	1
Synapsin-1	P17600	1
Coagulation factor V	P12259	1
Transmembrane channel-like protein 6	Q7Z403	1
Multimerin-1	Q13201	1
Vasodilator-stimulated phosphoprotein	P50552	1
Actin, cytoplasmic 2	P63261	1
Dermcidin	P81605	1
Putative serum amyloid A-3 protein	P22614	1
Apolipoprotein A-II	P02652	1
LIM and SH3 domain protein 1	Q14847	1
Corticotropin-lipotropin	P01189	1
ADP-ribosylation factor-like protein 6-interacting protein 4	Q66PJ3	1
Antithrombin	Q8IZZ8	1
Alpha-2-antiplasmin	P08697	1
Chondroitin sulfate proteoglycan 4	Q6UVK1	1
Gelsolin	P06396	1
Apolipoprotein L1	O14791	1

action [42]. Zyxin is a focal-adhesion-associated phosphoprotein with an apparent molecular weight of 82–84 kDa. It has been reported that zyxin expression plays a role in the motility of HCC cells, cell migration, and intravasation through its action on actin cytoskeleton [43]. Zyxin promoted cell dissemination as a part of the integrin-signaling pathway [43].

The fibrinogen and coagulation factor XIII A are

components of the blood coagulation cascade. The coagulation process involves thrombin-dependent cleavage of fibrinogen with the release of fibrinopeptide α . Fibrinogen is the most abundance source of peptides in our study and was also found to be associated with the presence of cancer in other studies [31,43,44]. We identified ladders of N- or C-terminal truncations of serum peptides as reported previously by several groups [14,

39,35]. The most likely explanation for the presence of these peptides is the presence of different exoprotease activities superimposed on an aberrant clotting process [14,24,45]. Our study was conducted in serum; however, we still observed the ladder of peptides derived from fibrinogen α when we analyzed EDTA plasma from disease free controls. Cancer is known to be associated with disorders of hemostasis and it is still unclear how much fragmentation occurs *in vivo* and what are the functional implications [46–50].

The spectrum in Fig. 1 is consistent with the fragmentation of biliverdin diglucuronide. Biliverdin is a product of heme breakdown. Biliverdin reductase reduces biliverdin to water insoluble bilirubin which is conjugated with glucuronic acid, mainly in the liver, by the enzyme UDP-glucuronyltransferase and excreted. Several authors pointed out that bilirubin is likely to play important physiological functions because biliverdin is water soluble and could be directly excreted. It was already shown that bilirubin has antioxidant [51] and immunomodulatory [52] functions. Bilirubin diglucuronide is a sign of liver injury; biliverdin diglucuronide is possibly an oxidation product of bilirubin diglucuronide reflective of liver injury [53]. We observed an increase in biliverdin diglucuronide in HCC compared to population controls [19–21] but the difference was not detected compared to cirrhosis controls [19–21].

In conclusion, we identified peptides and a metabolite associated with the presence of HCC by mass spectrometric sequencing. The peptides of complement C3, complement C4 and zyxin likely derive from a complex exopeptidase activity superimposed on differentially abundant peptides related to HCC. These peptides could potentially serve as markers of HCC; however, association of similar peptides with other diseases and cancers dictates a very cautious approach.

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