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A Quantitative Proteomic Approach for Identification of Potential Biomarkers in Hepatocellular Carcinoma

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Hepatocellular carcinoma (HCC) is the fifth most common cancer worldwide. In this study, our objective was to identify differentially regulated proteins in HCC through a quantitative proteomic approach using iTRAQ. More than 600 proteins were quantitated of which 59 proteins were overexpressed and 92 proteins were underexpressed in HCC as compared to adjacent normal tissue. Several differentially expressed proteins were not implicated previously in HCC. A subset of these proteins (six each from upregulated and downregulated groups) was further validated using immunoblotting and immunohistochemical labeling. Some of the overexpressed proteins with no previous description in the context of HCC include fibroleukin, interferon induced 56 kDa protein, milk fat globule-EGF factor 8, and myeloid-associated differentiation marker. Interestingly, all the enzymes of urea metabolic pathway were dramatically downregulated. Immunohistochemical labeling confirmed differential expression of fibroleukin, myeloid associated differentiation marker and ornithine carbamoyl transferase in majority of HCC samples analyzed. Our results demonstrate quantitative proteomics as a robust discovery tool for the identification of differentially regulated proteins in cancers.

Keywords: fibroleukin • myeloid-associated differentiation marker • HCV • iTRAQ • liver • mass spectrometry • strong cation exchange chromatography • urea cycle

Introduction

Hepatocellular carcinoma (HCC) is the fifth most common primary malignancy of the liver. It is often diagnosed in advanced stages resulting in a very high mortality rate. In the United States alone, 17 000−20 000 new cases are reported every year, and there is evidence suggesting that the incidence is increasing.[1−3] HCC is often seen in association with cirrhosis, and additional risk factors include hepatitis B (HBV) and hepatitis C (HCV), alcohol, aflatoxin, family history, and smoking.

Early diagnosis of HCC is known to improve the outcome. Improvements in imaging modalities have increased sensitivity, but at the cost of specificity. Currently available biomarkers, however, lack adequate sensitivity or specificity. Alpha-fetoprotein (AFP) is the most widely used biochemical blood test for HCC, which is elevated in less than 60% of patients.[4] Increased levels of AFP are common in patients with chronic hepatitis decreasing the utility of this test for surveillance purposes. Other potential biomarkers have been identified, although the majority of them have not been validated prospectively for clinical purposes.[5] Transcriptomic analyses of HCC suggest that it is a complex disease with numerous molecular alterations.[2,6,7]Thus, it is unlikely that a single biomarker will be adequate to monitor HCC and perhaps a panel of biomarkers is needed for early detection in high-risk population or when the imaging study is nonconfirmatory. Global profiling of HCC using quantitative proteomics would provide several potential markers differentially expressed in cancers. Although earlier studies have reported differentially expressed proteins in

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HCC, they have several limitations including lack of comprehensive and quantitative information.[8−10]

Isobaric tags for relative and absolute quantification (iTRAQ) is a set of amine-specific isobaric tags for multiplexed relative quantitation of proteins by mass spectrometry.[11] iTRAQ based quantitative proteomics is a promising approach for global comparison of protein expression in relatively small amount of samples. iTRAQ method has been employed in many cancer biomarker studies such as analysis of saliva in breast cancer,[12] human prostate cancer cell lines,[13] and chronic myeloid leukemia[14] and in breast cancer derived cells.[15]

In the current study, we have carried out global proteomic profiling of HCC and identified several differentially expressed proteins. Further, a subset of proteins showing greater than 3 fold difference in expression were validated by Western blotting and immunohistochemical labeling of tissue microarrays.

Materials and Methods

Tissue Samples. Liver tissue samples were procured after obtaining Institutional Review Board approval. Fresh tissues were collected at the time of surgery from patients with HCV associated HCC. Tumor and adjacent non-neoplastic tissues were determined by an experienced pathologist. Same sections of the tissue were formalin embedded and were used for both confirmation of tumor and nontumor regions and validation of iTRAQ data. Formalin fixed and paraffin embedded tissue microarrays (TMA) (thickness, 0.5 μM and diameter, 1.5 mm) were used for immunohistochemical labeling. Tissue microarrays obtained from Imgenex consisted of 13 metastatic tissues along with 46 poor to well differentiated hepatocellular carcinoma from patients in the age group of 40 to 72 years (Cat. No. IMH-318) and 59 noncancer tissues (Cat. No. IMH-342). The tissue microarrays from Creative Biolabs (Cat, No. CBL-TMA-070) consisted of 15 well differentiated, 29 moderately differentiated, and 12 poorly differentiated hepatocellular carcinoma from patients in the age group of 35−77 years and 20 noncancer tissues in triplicates (CBL-TMA-076).

iTRAQ Labeling and SCX Fractionation. Tumor and adjacent noncancerous liver tissue from HCC patient were used for iTRAQ labeling experiment. Ten milligrams of liver tissue lysates homogenized in 0.5% SDS and protease inhibitors (PMSF and EDTA) using Dounce homogenizer followed by sonication. Samples were normalized based on protein concentration. Trypsin digestion and iTRAQ (Applied Biosystems Cat. No. 4352135) labeling was carried out according to manufacturer's protocol using the reagents provided unless otherwise mentioned. Briefly, 80 μg of each lysate was treated with 2 μL of reducing agent (tris(2-carboxyethyl) phosphine (TCEP)) at 60 °C for 1 h and alkylated with 1 μL of cysteine blocking reagent, methyl methanethiosulfonate (MMTS)) for 10 min at room temperature. Protein sample was digested using sequencing grade trypsin (Promega) (1:10) for 16 h at 37 °C.[15] Peptides from each sample in a final volume of 40 μL were labeled with one of the four iTRAQ reagents at room temperature. After labeling tryptic digests from noncancerous or cancerous tissue samples, the peptides were mixed and fractionated on strong cation exchange chromatography on PolySULFOETHYL A column (PolyLC, Columbia, MD) (100 × 2.1 mm, 5 μm particles with 300 Å pores) using an LC Packing HPLC system connected to a Probot fraction collector. Thirty-nine SCX fractions (0.5 mL) were collected from a 0−350 mM KCl gradient in the presence of 10 mM potassium phosphate buffer (pH 2.85), containing 25% acetonitrile for 70 min at a flow rate of 0.2 mL/min. Solvent A contained 10 mM potassium phosphate buffer, pH 2.85, 25% acetonitrile and solvent B contained 10 mM potassium phosphate buffer, 350 mM KCl, pH 2.85, 25% acetonitrile. The fractions were dried and reconstituted in 10 μL of 2% trifluoroacetic acid or stored at −80 °C until mass spectrometry analysis.

Mass Spectrometry and Protein Quantitation. LC−MS/MS analysis of the sample was carried out using reversed-phase liquid chromatography (RP-LC) interfaced with a quadrupole time-of-flight mass spectrometer (QSTAR/pulsar, Applied Biosystems). RP-LC system (Agilent 1100 system) consisted of a trap column (75 μm × 3 cm, C_{18} material 5−10 μm, 120 Å) and an analytical column (75 μm × 10 cm, C_{18} material 5 μm, 120Å) fitted with an emitter tip 8 μm (New Objective, Woburn, MA). LC−MS/MS data was acquired by online analysis of peptides eluted using 5−40% acetonitrile in 0.1% formic acid for 30 min with a flow rate of 300 nL/min. The MS spectra were acquired in a data dependent manner from m/z 350 to 1200 Da targeting three most abundant ions in the survey scan and those ions selected were excluded from MS/MS for 45s. Twenty percent higher collision energy was applied during MS/MS scan with a charge state dependent collision energy selection criteria. Proteinpilot software v2.0.1 (Applied Biosystems MDS SCIEX) was used for identification and quantitation of proteins. The data from 39 LC−MS/MS analyses on QStar was searched against NCBI RefSeq database version 26 containing human 39 380 protein entries. Peptide and protein identification was carried out according to the Molecular and Cellular Proteomics guidelines.[16] Paragon algorithm in Proteinpilot was used for peptide identification and subsequently searched results were processed by Pro Group algorithm. Search parameters included iTRAQ labeling at N-terminus and lysine residues, cysteine modification by methyl methanethiosulfonate (MMTS), digestion by trypsin. Isoform specific identification and quantitation was carried out by selecting peptides distinct to each form and excluding all shared peptides from quantitation. Proteins identified with >95% confidence or Protscore >1.3 were used for further analysis.

Western Blotting. Tumor and normal tissues were homogenized in 0.5% SDS containing protease inhibitor mixture (Complete: Roche Applied Science). Eighty micrograms of protein from each tissue was transferred electrophoretically onto nitrocellulose membrane (Protran, Whatman). After the transfer of protein, nitrocellulose membrane was blocked with 5% BSA in phosphate-buffered saline with 0.1% Tween 20 (PBS-T) for 1 h at room temperature. The following antibodies were used: fibroleukin (1:2000, sc-30869, Santa Cruz Biotech.), vitamin-D binding protein (1:1000, sc-32899, Santa Cruz Biotech), filamin 1 (1:500, sc-17749, Santa Cruz Biotech), FHL1B (1:1000, 18−003−42516, Genway), fibrillin 2 (1:500, ab21619, Novus), talin 1 (1:500, ab11188, Abcam), cystatin B (1:1000, AF1408 R&D systems), ornithine carbamoyl transferase (1:500, HPA000243, Sigma), prostatic binding protein (1:500, 4742, Cell Signaling), fatty acid binding protein 1 (1: 500, sc-50380, Santa Cruz Biotech), carbamoyl phosphate synthetase 1 (1: 500, sc-30060, Santa Cruz Biotech), Arginase 1 (1:500, HPA003595, Sigma), and microsomal epoxide hydrolase (1:500, E93220, BD Biosciences). The membranes were probed with primary antibody followed by horseradish peroxidase-conjugated antibody and developed using enhanced chemiluminescence detection according to the manufacturer's instructions (Amersham Biosciences).

Immunohistochemical Labeling. Immunohistochemical (IHC) labeling[17] was also performed on liver tissues from the same patient's sample used for the proteomic experiment. For the screening purposes liver cancer tissue microarrays containing large number of HCC and noncancer liver tissue samples were used. Novel candidates selected for immunohistochemistry analysis included fibroleukin, myeloid-associated differentiation marker (IMG-5908A, Imgenex), vitamin-D binding protein and ornithine carbamoyl transferase. Immunohistochemical labeling of fibroleukin and ornithine carbamoyl transferase was performed on IMH-318 and IMH-342 tissue microarrays. IHC for myeloid-associated differentiation marker was carried out using tissue microarrays from Creative Biolabs. The Envision kit (DAKO) was used according to the manufacturer's instructions. In brief, formalin fixed paraffin embedded tissue sections were deparaffinized and antigen retrieval was performed for 20 min in 0.01 mol/L of sodium citrate buffer. Endogenous peroxidase was quenched using hydrogen peroxide. The sections were incubated with primary antibody (dilution 1:100). After rinsing with wash buffer, the slides were incubated with HRP conjugated appropriate secondary antibody. The signal was developed using Dako chromogen supplied for peroxidase. Tissue sections were observed using Nikon DS-Fi1, microscope operated using NIS-Elements F package. The immunohistochemical labeling was assessed by an experienced liver pathologist and staining intensity was scored as negative (0), weak (1+), moderate (2+), and strong (3+). The distribution of staining of cancer cells was scored as 0 (less than 5% of cells staining), 1+ (5−30% of cell staining), 2+ (31−60% of cells staining) and 3+ (greater than 60% of cells staining). The intensity and distribution scores were then summed for each case.

Results

iTRAQ Labeling and Protein Quantitation. Our goal was to identify differentially expressed proteins in hepatocellular carcinoma and to subsequently validate a subset of these potential biomarkers. For the discovery phase of our study, we selected the tumor and noncancerous liver tissue from the same patient diagnosed with HCV-related HCC. Lysates of tumor and noncancerous liver tissues were labeled with iTRAQ reagents as shown in Figure 1. Technical replicates were prepared by labeling noncancer tissue derived peptides with 114 and 115 and HCC derived peptides with 116 and 117 iTRAQ labels. The data from a total of 40 504 MS/MS spectra (nonempty) generated by LC−MS/MS analysis of 39 SCX fractions were searched against the human RefSeq database using Proteinpilot. Based on the identification and quantitation criteria set in Proteinpilot, a protein is reported if a quantitation ratio is obtained using at least two unique peptides with "unused" confidence cutoff (ProtScore) >1%. However, manual inspection of spectra was carried out wherever identification was supported by less than 70% confidence values. Using ProtScore >1.3 (95%) cutoff, 639 proteins were identified from 10 270 distinct peptides (20 833 MS/MS spectra). Using ProtScore >1.0 (Cutoff >90% confidence) we found 22 additional proteins, which were manually validated. The complete list of these proteins can be found in Supplementary Table 1 (Supporting Information). Bias correction was applied to account for normalization errors.

Representative peptide MS/MS spectra and reporter ions of 8 differentially expressed proteins are shown in Figure 2. The fold changes were calculated from the ratio of intensity

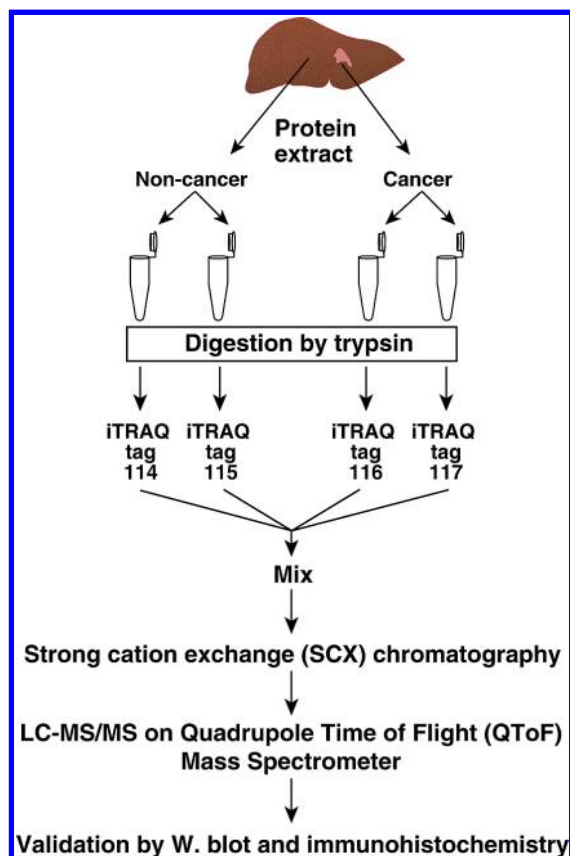

Figure 1. Quantitative proteomic analysis of hepatocellular carcinoma. Homogenates were prepared from the tumor and adjacent noncancerous liver tissues from a diagnosed case of HCC. The protein samples (80 μg from each sample) were digested using trypsin, and peptides were labeled with iTRAQ reagents. Labeled peptides were combined and fractionated by strong cation exchange chromatography. Thirty-nine fractions (0.5 mL) were obtained and analyzed by LC−MS/MS.

of iTRAQ reporter ions obtained for tumor derived peptides to those derived from adjacent noncancerous tissue. Using 3-fold as a stringent cutoff to designate up or downregulated proteins, we observed that 59 proteins were upregulated and 92 proteins were downregulated in HCC (Table 1 and Table 2). To our knowledge, this is the first large-scale quantitative proteomics profiling of HCC tissue revealing several novel and known differentially regulated proteins. Importantly, we were able to identify many proteins that have been previously reported to be upregulated in HCC. Proteins and peptides identified in this study have also been deposited in Human Proteinpedia, a publicly available portal for sharing and integration of human proteomic data,[18] where the MS/MS spectra of peptides from the proteins identified in this study can be visualized.

Validation of Quantitative Proteomics Results. A large number of differentially expressed proteins identified in this study have not previously been described in HCC (See table 1 for a list of upregulated proteins). To validate our findings using Western blot analysis, we selected 6 upregulated proteins encoded by FGL2, GC, FLNA, FHL1, FBN2, and TLN1 genes and 6 downregulated proteins encoded by OTC, PEBP1, FABP1, CPS1, ARG1 and EPHX1 genes (Figure 3). The results from Western blot analysis were in accordance with iTRAQ results in case of both upregulated and downregulated proteins.

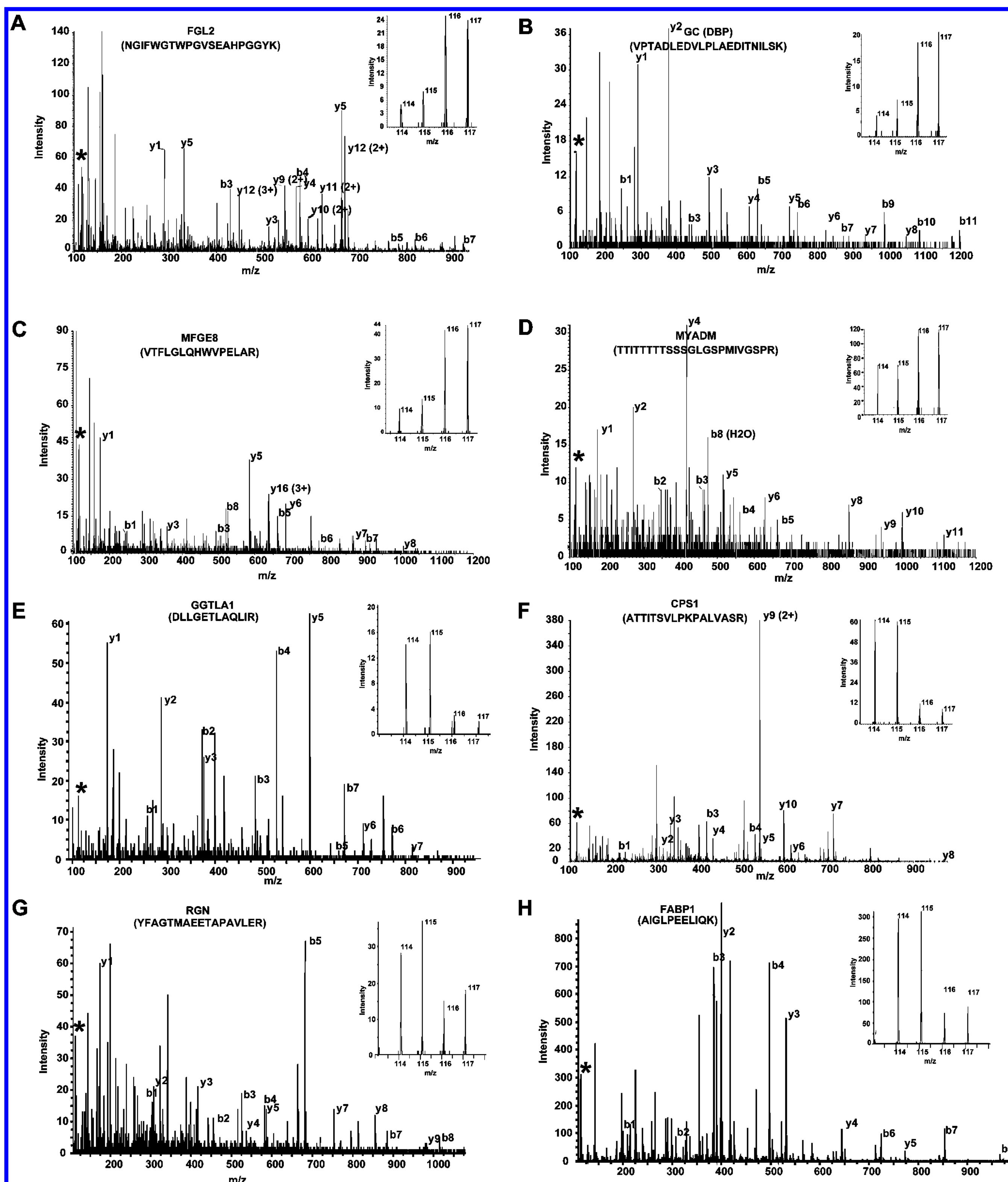

Figure 2. Quantitation by iTRAQ. (A–D) MS/MS spectra of representative peptides from upregulated proteins; fibroleukin (FGL2), vitamin-D binding protein (GC), milk fat globule-EGF factor 8 (MFGE8), and myeloid-associated differentiation marker (MYADM), respectively. (E–H) MS/MS spectra of gamma-glutamyltransferase-like activity 1 (GGTLA1), carbamoyl-phosphate synthetase 1, (CPS1), regucalcin (RGN), and fatty acid binding protein 1 (FABP1), respectively. (Insets) Relative intensity of reporter ions (*m/z*; normal 114, 115 and tumor 116, 117) from MS/MS fragmentation.

The proteomics results were further validated in the same tissues by immunohistochemical labeling of three upregulated proteins (fibroleukin, myeloid-associated differentiation marker and vitamin-D binding protein) and one downregulated protein (ornithine carbamoyl transferase) (Figure 4). While fibroleukin showed a stromal staining pattern in cancer tissue, both myeloid-associated differentiation marker and vitamin-D binding protein showed a hepatocellular staining pattern. We

Table 1. List of Upregulated Proteins Identified with No Previously Published Association with Hepatocellular Carcinoma

no.	RefSeq accession #	Gene symbol	Protein name	iTRAQ ratio (tumor/nontumor)
1	NP_001440.2	FHL1	Four and a half LIM domains 1	9.6
2	NP_001001670.1	FLJ46321	Hypothetical protein LOC389763	9.4
3	NP_001539.3	IFIT1	Interferon-induced protein with tetratricopeptide repeats 1 isoform 2	8.5
4	NP_001990.2	FBN2	Fibrillin 2	8.4
5	NP_006673.1	FGL2	Fibroleukin	8.2
6	NP_002209.2	ITIH4	Interalpha (globulin) inhibitor H4	6.6
7	XP_001131713.1	LOC730410	PREDICTED: similar to HLA class I histocompatibility antigen, B-18 alpha chain	6.5
8	NP_001701.2	CFB	Complement factor B	6.1
9	NP_000925.2	SERPINF2	Alpha-2-plasmin inhibitor	6.1
10	NP_001018657.1	MYADM	Myeloid-associated differentiation marker	5.6
11	NP_001026862.1	LRRC17	Leucine rich repeat containing 17 isoform 1	5.6
12	NP_001076.2	SERPINA3	Serpin peptidase inhibitor, clade A, member 3	5.0
13	NP_000468.1	ALB	Albumin	4.5
14	NP_005919.1	MFGE8	Milk fat globule-EGF factor 8 protein	4.4
15	NP_001634.1	APOA2	Apolipoprotein A-II	4.4
16	NP_004361.3	COL12A1	Collagen, type XII, alpha 1 long isoform	4.1
17	NP_036544.1	H2AFV	H2A histone family, member V isoform 1	4.1
18	NP_001210.1	CALU	Calumenin	4.0
19	NP_000030.1	APOA1	Apolipoprotein A-I	4.0
20	NP_001918.3	DES	Desmin	3.9
21	NP_000292.1	PLG	Plasminogen	3.8
22	NP_001613.1	AHSG	Alpha-2-HS-glycoprotein	3.8
23	NP_005264.2	GNB2	Guanine nucleotide-binding protein, beta-2 subunit	3.8
24	NP_004326.1	BST2	Bone marrow stromal cell antigen 2	3.7
25	NP_068758.2	FKBP10	FK506 binding protein 10, 65 kda	3.7
26	NP_002499.2	NID1	Nidogen 1	3.7
27	NP_055129.2	DDX58	DEAD/H (Asp-Glu-Ala-Asp/His) box RIG-I	3.6
28	NP_005132.2	FGB	Fibrinogen, beta chain	3.6
29	NP_114437.2	EMILIN2	Elastin microfibril interfacer 2	3.5
30	NP_997637.1	PRKAR1A	Camp-dependent protein kinase, regulatory subunit alpha 1	3.4
31	NP_000574.2	GC	Vitamin D-binding protein	3.4
32	NP_000499.1	FGA	Fibrinogen, alpha isoform alpha-E	3.4
33	NP_002207.2	ITIH2	Interalpha globulin inhibitor H2	3.4
34	NP_002061.1	GNAI2	Guanine nucleotide binding protein (G protein), alpha inhibiting activity 2	3.3
35	NP_005520.4	HSPG2	Heparan sulfate proteoglycan 2	3.3
36	NP_001760.1	CD9	CD9 antigen	3.3
37	NP_001074419.1	MYO1C	Myosin IC isoform b	3.2
38	NP_005134.1	HP	Haptoglobin	3.2
39	NP_001002235.1	SERPINA1	Serine (or cysteine) proteinase inhibitor, clade A, member 1	3.2
40	NP_068656.2	FGG	Fibrinogen, gamma chain isoform gamma-B	3.2
41	NP_063940.1	DIABLO	Diablo isoform 1	3.1
42	NP_444253.3	MYLK	Myosin light chain kinase isoform 1	3.1
43	NP_005511.1	HNRNPH1	Heterogeneous nuclear ribonucleoprotein H1	3.1
44	NP_001072990.1	KTN1	Kinectin 1 isoform b	3.1
45	NP_005794.1	FLOT1	Flotillin 1	3.1
46	NP_068800.1	PGM5	Phosphoglucomutase 5	3.0
47	NP_001728.1	C9	Complement component 9	3.0
48	NP_002453.1	MX1	Myxovirus resistance protein	3.0

observed loss of expression of ornithine carbamoyl transferase in cancer cells as compared to the corresponding normal.

Validation of Quantitative Proteomics Results Using Tissue Microarrays. Prevalence of overexpression of fibroleukin, myeloid-associated differentiation marker was determined by staining HCC tissue microarrays. Fibroleukin was overexpressed in 33/56 (59%) of HCC cases with the total score ranging from 2 to 5 whereas in 8/56 (14%) of non-HCC cases, the total score was 2 (Figure 5). Myeloid-associated differentiation marker protein showed distinct staining profile in cancers as compared to normal liver tissue. We identified myeloid-associated differentiation marker as a promising biomarker because antibodies against this protein stained 34 out of 53 HCC samples (64%) but in none of the noncancerous liver tissue ($n = 20$) (Figure 5). This protein showed 63% sensitivity and 100% specificity. Ornithine carbamoyl transferase (OTC), one of the underexpressed proteins in our proteomic screen was also screened using tissue microarrays. Expression of OTC was significantly decreased in 24/59 (42%) of HCC tissues, among them 15 cases showed negative staining, 8 sections showed less than 5% staining. Sixteen of remaining 25 HCC cases showed moderate decrease in expression of OTC. OTC expression was highly detectable (score >5) in all the normal cases analyzed (59/59).

Discussion

Proteins Upregulated in HCC. This proteomics strategy was able to identify and quantitate differentially expressed proteins which are previously described in several HCC biomarker analysis studies. Among the proteins found to be upregulated in this study, eleven of them were previously shown to be associated with HCC (Table 3). SPARC like 1 (hevin) shares 60% sequence similarity with secreted protein acidic and rich in cysteine (SPARC) has been shown to be overexpressed in HCC.[19] Although, SPARC like 1 is barely expressed in normal

Table 2. List of Downregulated Proteins Identified in Hepatocellular Carcinoma

no.	RefSeq accession #	Gene symbol	Protein name	iTRAQ ratio (tumor/non- tumor)	ratio from published microarray studies[a]
1	NP_000522.3	OTC	Ornithine carbamoyltransferase	0.1	N/A
2	NP_004112.1	GGTLA1	gamma-glutamyltransferase-like activity 1	0.1	Increased
3	NP_000932.2	POR	Cytochrome P450 reductase	0.1	Decreased
4	NP_059488.2	CYP3A4	Cytochrome P450, subfamily IIIA, 4	0.2	N/A
5	NP_000215.1	KRT18	Keratin 18	0.2	Decreased
6	NP_001905.1	CYB5A	Cytochrome *b*-5 isoform 2	0.2	N/A
7	NP_000660.1	ADH1C	Class I alcohol dehydrogenase, gamma subunit	0.2	N/A
8	NP_002890.1	RBP1	Retinol binding protein 1, cellular	0.2	Decreased
9	NP_000839.1	GSTM2	Glutathione S-transferase M2	0.2	N/A
10	NP_005850.1	PURA	Purine-rich element binding protein A	0.2	N/A
11	NP_057058.2	CRYL1	Lambda-Crystallin	0.2	N/A
12	NP_000182.2	HMGCL	3-hydroxymethyl-3-methylglutaryl-Coenzyme A lyase	0.2	Decreased
13	NP_001866.2	CPS1	Carbamoyl-phosphate synthetase 1, mitochondrial	0.2	Increased
14	NP_976059.1	BDH1	3-hydroxybutyrate dehydrogenase	0.2	Decreased
15	NP_002970.2	SCP2	Sterol carrier protein 2 isoform 1 proprotein	0.2	Decreased
16	NP_001598.1	ACAA1	Acetyl-Coenzyme A acyltransferase 1	0.2	N/A
17	NP_000661.2	ADH4	Class II alcohol dehydrogenase 4 pi	0.2	Decreased
18	NP_001434.1	FABP1	Fatty acid binding protein 1, liver	0.2	Decreased
19	NP_000041.2	ASS1	Argininosuccinate synthetase	0.2	Decreased
20	NP_056348.2	DAK	Dihydroxyacetone kinase 2	0.2	N/A
21	NP_995317.1	AKR1C2	Aldoketo reductase family 1, C2	0.2	N/A
22	NP_001900.1	CTSD	Cathepsin D	0.2	N/A
23	NP_002264.1	KRT8	Keratin 8	0.2	Decreased
24	NP_000552.2	GSTM1	Glutathione S-transferase M1 isoform 1	0.2	Decreased
25	NP_000657.1	ACY1	Aminoacylase 1	0.2	N/A
26	NP_000036.2	ARG1	Arginase, type I	0.2	Decreased
27	NP_003555.1	TAGLN2	Transgelin 2	0.2	Increased
28	NP_002558.1	PEBP1	Prostatic binding protein	0.2	Decreased
29	NP_003303.2	TST	Thiosulfate sulfurtransferase	0.2	Decreased
30	NP_001020366.1	CES1	Carboxylesterase 1 isoform a	0.2	Decreased
31	NP_001743.1	CAT	Catalase	0.2	Decreased
32	NP_005509.1	HMGCS2	3-hydroxy-3-methylglutaryl-Coenzyme A synthase 2	0.2	N/A
33	NP_064695.2	AKR1B10	Aldo-keto reductase family 1, member B10	0.2	N/A
34	NP_000420.1	MAT1A	Methionine adenosyltransferase I, alpha	0.2	N/A
35	NP_008825.4	FMO3	Flavin containing monooxygenase 3 isoform 1	0.2	N/A
36	NP_000680.2	ALDH1A1	Aldehyde dehydrogenase 1A1	0.2	N/A
37	NP_004554.2	PCK2	Mitochondrial phosphoenolpyruvate carboxykinase 2 isoform 1	0.2	Decreased
38	NP_001986.2	ACSL1	Acyl-coa synthetase long-chain family member 1	0.2	Decreased
39	NP_005023.2	PLS3	Plastin 3	0.2	N/A
40	NP_002141.1	HPD	4-hydroxyphenylpyruvate dioxygenase	0.2	Decreased
41	NP_061833.1	GNMT	Glycine N-methyltransferase	0.2	N/A
42	NP_000681.2	ALDH2	Mitochondrial aldehyde dehydrogenase 2	0.2	N/A
43	NP_060084.2	BHMT2	Betaine-homocysteine methyltransferase 2	0.2	Decreased
44	NP_001026976.1	ALDH3A2	Aldehyde dehydrogenase 3A2 isoform 1	0.2	N/A
45	NP_004083.2	ECHS1	Mitochondrial short-chain enoyl-coenzyme A hydratase 1	0.2	Decreased
46	NP_002070.1	GOT1	Aspartate aminotransferase 1	0.3	Decreased
47	NP_004365.1	COX6C	Cytochrome c oxidase subunit vic proprotein	0.3	Increased
48	NP_000498.2	FBP1	Fructose-1,6-bisphosphatase 1	0.3	Decreased
49	NP_057370.1	DCXR	Dicarbonyl/L-xylulose reductase	0.3	N/A
50	NP_001473.1	GATM	L-arginine:glycine amidinotransferase	0.3	Decreased
51	NP_006420.1	CCT7	Chaperonin containing TCP1, subunit 7 isoform a	0.3	N/A
52	NP_001944.1	TYMP	Endothelial cell growth factor 1 (platelet-derived)	0.3	N/A
53	NP_005887.2	IDH1	Isocitrate dehydrogenase 1 (NADP+), soluble	0.3	N/A
54	NP_061978.5	TNXB	Tenascin XB isoform 1	0.3	N/A
55	NP_001893.2	CTH	Cystathionase isoform 1	0.3	Decreased
56	NP_003725.1	AOC3	Amine oxidase, copper containing 3	0.3	Decreased
57	NP_000454.1	UGT1A1	UDP glycosyltransferase 1 family, A1	0.3	N/A
58	NP_000895.1	NQO2	NAD(P)H dehydrogenase, quinone 2	0.3	N/A
59	NP_001065.1	UGT2B7	UDP glycosyltransferase 2 family, B7	0.3	Decreased
60	NP_000659.2	ADH1B	Alcohol dehydrogenase 1B (class I), beta	0.3	Decreased
61	NP_003281.1	TPM4	Tropomyosin 4	0.3	N/A
62	NP_000089.1	CPT2	Carnitine palmitoyltransferase II	0.3	Decreased
63	NP_037523.2	DMGDH	Dimethylglycine dehydrogenase	0.3	N/A
64	NP_001009186.1	CCT6A	Chaperonin containing TCP1, subunit 6A isoform b	0.3	N/A
65	NP_001809.2	AKR1C4	Aldo-keto reductase family 1, member C4	0.3	N/A
66	NP_000026.2	ALDOB	Aldolase B	0.3	Decreased
67	NP_002957.1	S100A10	S100 calcium-binding protein A10	0.3	Increased
68	NP_004036.1	ATOX1	Antioxidant protein 1	0.3	Increased
69	NP_612402.1	REEP6	Polyposis locus protein 1-like 1	0.3	Decreased
70	NP_002188.1	ACO1	Aconitase 1	0.3	Decreased
71	NP_000021.1	AGXT	Alanine-glyoxylate aminotransferase	0.3	Decreased
72	NP_005262.1	GLUD1	Glutamate dehydrogenase 1	0.3	Decreased
73	NP_004674.1	RGN	Regucalcin	0.3	Decreased
74	NP_005557.1	LDHA	Lactate dehydrogenase A	0.3	Decreased

Table 2. Continued

no.	RefSeq accession #	Gene symbol	Protein name	iTRAQ ratio (tumor/non- tumor)	ratio from published microarray studies[a]
75	NP_001073332.1	DBI	Diazepam binding inhibitor isoform 2	0.3	N/A
76	NP_002328.1	LRPAP1	Low density lipoprotein receptor-related protein associated 1	0.3	N/A
77	NP_036344.1	PTGR1	NADP-dependent leukotriene B4 12-hydroxydehydrogenase	0.3	N/A
78	NP_009193.2	PARK7	DJ-1 protein	0.3	N/A
79	NP_116139.1	ABHD14B	Abhydrolase domain containing 14B	0.3	N/A
80	NP_006102.1	ACAA2	Acetyl-coenzyme A acyltransferase 2	0.3	Decreased
81	NP_060015.1	HAO1	Hydroxyacid oxidase 1	0.3	Decreased
82	NP_000356.1	TPI1	Triosephosphate isomerase 1	0.3	N/A
83	NP_001748.1	CBR1	Carbonyl reductase 1	0.3	Decreased
84	NP_000764.1	CYP2E1	Cytochrome P450, family 2, subfamily E, 1	0.3	N/A
85	NP_665683.1	GSTA1	Glutathione S-transferase A1	0.3	N/A
86	NP_001350.1	DECR1	2,4-dienoyl coa reductase 1	0.3	Decreased
87	NP_004823.1	GSTO1	Glutathione-S-transferase omega 1	0.3	Decreased
88	NP_055066.1	SLC25A13	Solute carrier family 25, member 13 (citrin)	0.3	N/A
89	NP_001975.1	ESD	Esterase D/formylglutathione hydrolase	0.3	Decreased
90	NP_005947.2	MTHFD1	Methylenetetrahydrofolate dehydrogenase 1	0.3	Decreased
91	NP_660202.2	NAPRT1	Nicotinate phosphoribosyltransferase domain containing 1	0.3	N/A
92	NP_000166.2	GPI	Glucose phosphate isomerase	0.3	Increased
93	NP_000111.1	EPHX1	Epoxide hydrolase 1, microsomal	0.4	Decreased

[a] Oncomine.

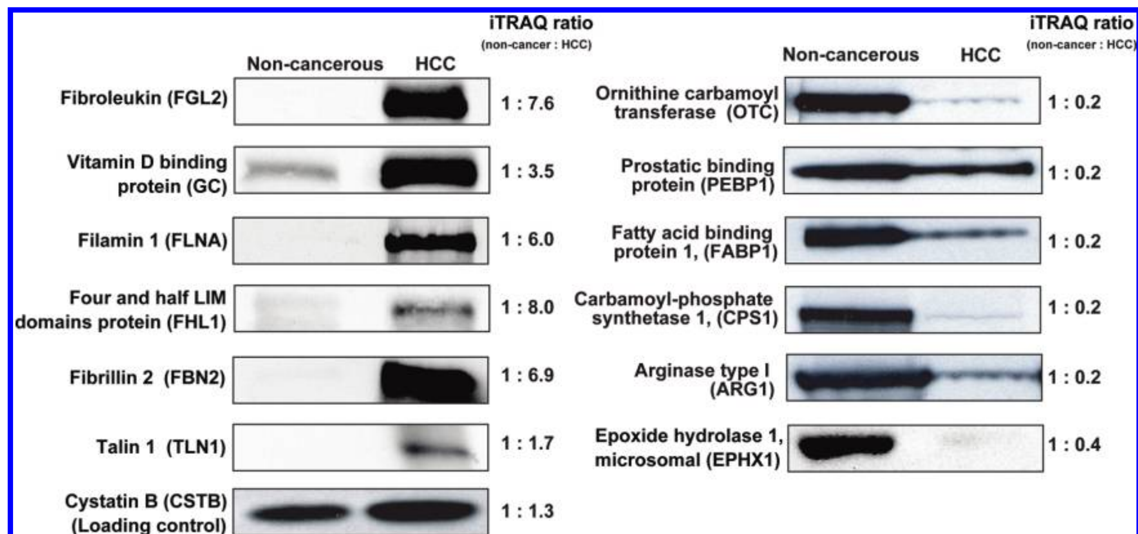

Figure 3. Validation of iTRAQ results by Western blotting. Liver homogenates used for the quantitative proteomic analysis were resolved by SDS-PAGE, subsequently electroblotted onto nitrocellulose membrane and probed with specific antibodies as indicated. The corresponding ratios from the iTRAQ labeling experiments are also shown for comparison.

liver, significance of its overexpression in HCC is not well studied. Overexpression of tenascin C and type IV collagen induces a defective extracellular matrix pattern in hepatocellular cancer.[20] Other major overexpressed proteins in HCC were vitronectin and fibronectin, which are involved in cell migration and adhesion in HCC along with other basement membrane proteins such as collagen IV and laminin.[21−23]

The present study reveals many novel HCC associated proteins. Fibroleukin (also known as fibrinogen-like protein 2), a secreted protein similar to fibrinogen beta and gamma with procoagulant activity,[24] was elevated 8-fold in HCC. Fibroleukin is associated with fibrin deposition and liver necrosis especially in human viral hepatitis.[25] It is also implicated in allograft rejection and adjunctive therapy to treat allograft rejection.[26] Vitamin D binding protein is involved in binding and transport of vitamin D and metabolites to target tissues and is found in blood, ascitic fluid, cerebrospinal fluid and bound to the cell surface. We found it to be upregulated 3.4-fold in HCC. Four and a half LIM domains 1 protein is a transcription factor and was upregulated 9.6 fold in HCC. It is widely expressed in skeletal muscle, liver and pancreas and has been implicated in differentiation, spreading and migration of muscle cells. To date, it has not been associated with any malignancy. Myeloid-associated differentiation marker is a protein with multiple transmembrane domains that was initially identified based on its high expression in myeloid progenitor cell lines. This protein has been shown to be upregulated during differentiation of hematopoietic cells or acute promyelocytic leukemia cells.[27] In our study, this protein was overexpressed 5.6-fold. Milk fat globule EGF factor 8 (MFGE8), a known marker of breast cancer, is secreted from activated macrophages and specifically binds to apoptotic cells enhancing the engulfment of apoptotic cells by phagocytes. In our study this protein was upregulated 4.4-fold. Interferon-induced protein with tetratricopeptide repeats 1 (IFIT1) is an interferon-induced protein. Increase in IFIT1 levels have been reported following adenovirus infec-

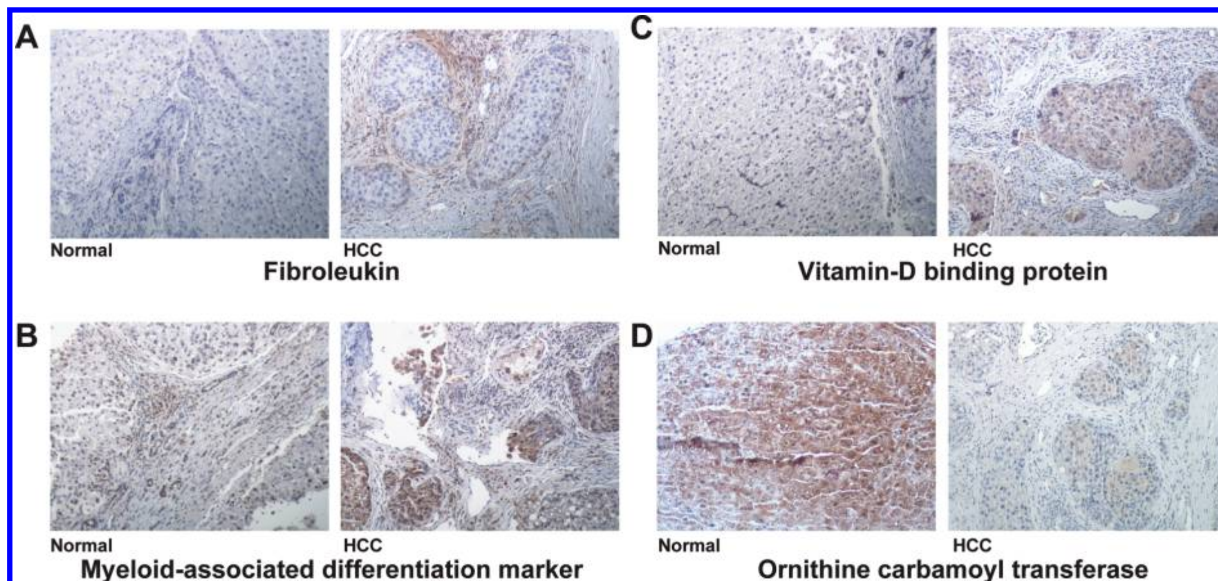

Figure 4. Confirmation of iTRAQ results using immunohistochemical labeling. Confirmation of upregulation of fibroleukin, myeloid-associated differentiation marker and vitamin D binding protein and downregulation of ornithine carbamoyl transferase in tissue samples used for iTRAQ analysis are shown. (A–D) Immunohistochemical labeling for fibroleukin, myeloid-associated differentiation marker, vitamin-D binding protein, and ornithine carbamoyl transferase, respectively (10× magnification).

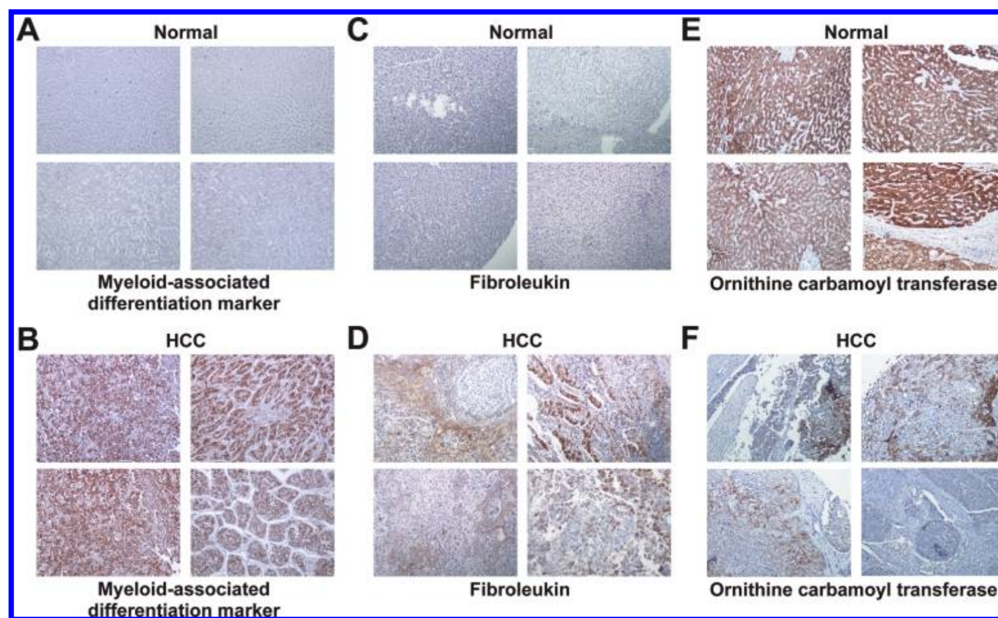

Figure 5. Validation of differential expression of myeloid-associated differentiation marker, fibroleukin, and ornithine carbamoyl transferase in HCC using tissue microarrays. IHC pictures (10× magnification) from four representative liver tissue sections, each of HCC and noncancerous tissues are shown. (A and B) IHC labeling for myeloid-associated differentiation marker in normal and HCC tissues, respectively. (C and D) IHC labeling for antifibroleukin in normal and HCC tissues, respectively. (E and F) IHC labeling for antiornithine carbamoyl transferase in normal and HCC tissues, respectively.

tion.[28] Moreover, IFIT1 is overexpressed in superficial basal cell carcinomas and cutaneous T-cell lymphomas.[29] In our study, this protein was upregulated 8.0-fold.

Proteins Downregulated in HCC. With a stringent cutoff of 3-fold downregulation (i.e., <0.3 ratio in tumor versus nontumor), we found that 92 proteins were downregulated in tumor tissue compared to noncancerous tissue. The functional implication of these proteins in the aggravation of the disease processes can be inferred by the classification of these proteins into groups involved in important molecular and biological processes. More than 90% of the proteins are involved in metabolic processes and a majority of them have catalytic activity. A significant number of proteins (30%) were involved in transporter activity. A list of downregulated proteins is shown in Table 2 and Supplementary Table 1 (Supporting Information). Major downregulated proteins included cathepsin D (0.2 fold), lysosomal aspartyl protease (0.2 fold), fatty acid binding protein 1 (0.2 fold), an important intracellular liver protein involved in lipid transport, and calponin homology domain protein (0.2 fold), which has not been well studied. Many calcium binding proteins such as S100 calcium-binding protein A10, calreticulin, regucalcin, and calpain were also found to be downregulated in HCC. Changes in calcium binding proteins have been studied in diverse pathological conditions including

Table 3. List of Identified Proteins That Were Previously Reported to Be Upregulated in Hepatocellular Carcinoma

	Gene symbol	Protein name	Reference	iTRAQ ratio in this study (tumor/nontumor)
1.	SPARCL1	SPARC-like 1	Lau CP, et al.[19]	9.9
2.	FN1	Fibronectin	Jagirdar, J et al.[37]	4.9
3.	VTN	Vitronectin	Kondoh N et al.[38]	4.5
4.	TNC	Tenascin C (Hexabrachion)	Zhao M, et al.[20]	4.3
5.	CAV1	Caveolin 1	Yerian LM, et al.[39]	4.2
6.	SOD3	Superoxide dismutase 3,extracellular	Takashima M et al.[40]	4.2
7.	TF	Transferrin	Suzuki Y et al.[41]	3.9
8.	LAMC1	Laminin gamma	Nakamura S et al.[42]	3.6
9.	HPX	Hemopexin	Darabi A et al.[43]	3.3
10.	C3	Complement component 3	Lee, IN et al.[44]	3.0
11.	COL6A1	Collagen, type VI, alpha 1	Zhao M, et al.[20]	3.2

cancer.[30,31] Prostatic binding protein (5-fold decrease in HCC) is a raf kinase inhibitor protein; an earlier study showed that expression of this protein is decreased in both cirrhotic liver and HCC as compared to normal liver.[32] Loss of raf kinase inhibitor protein expression was also found to be a key molecular event in colorectal cancer.[33] Although the significance of these findings remains currently unclear, these results demonstrate that proteomic approaches could also provide an insight into global derangements in important metabolic processes that occur in HCC.

Investigation of our large set of downregulated proteins showed that all five enzymes of urea cycle were significantly downregulated in HCC (Figure 6). Urea cycle is an essential metabolic pathway of liver for detoxification of ammonia that is metabolized by 5 enzymes. The status of all five enzymes in the urea metabolic pathway, reconstructed from KEGG database (http://www.genome.jp/kegg/)[34] is shown in Figure 6. Carbamoyl phosphate synthase (CPS1) and ornithine carbamoyl transferase are compartmentalized to mitochondria with CPS1 being the rate limiting enzyme in urea cycle. Overall, the expression level of carbamoyl phosphate synthase, ornithine carbamoyltransferase, argininosuccinate synthase, argininosuccinate lyase, and arginase were found to be dramatically reduced. An earlier study reported downregulation of CPS1 mRNA in HCC.[35] To our knowledge, however, this is the first study describing the status of all the enzymes of urea metabolic pathway using a proteomics approach. Three of the enzymes in intermediary metabolism, associated with urea cycle: cytosolic malate dehydrogenase (0.7 fold), aspartate amino transferase (0.3 fold), and fumarase (0.8 fold) were also downregulated in HCC. Analysis of downregulated proteins in Oncomine[36] (a cancer profiling database) shows a similar downregulation at the mRNA level in cancer versus noncancer (Table 2). Western blot analysis of CPS1, OTC, and arginase correlated with iTRAQ data. The immunohistochemical analysis of one of the urea cycle enzyme, ornithine carbamoyl transferase indicates the urea cycle enzymes are substantially downregulated across HCC samples. We have also observed similar results in gene expression studies using microarrays from the public repositories. This may be of significant interest, however further studies are needed to address the role of downregulation of urea cycle in HCC.

Early diagnosis of HCC improves prognosis, but currently available biochemical markers or imaging modalities either lack adequate sensitivity or specificity, especially in the presence of cirrhosis. Due to the complexity of the disease, a single, reliable biomarker is unlikely to be identified for sensitive and specific diagnosis of HCC. Quantitative proteomic profiling experiments offer an effective alternative for identifying differentially expressed proteins as potential biomarkers. In this study, we have identified many upregulated and downregulated proteins in HCC that may have pathological relevance as well as diagnostic potential. Studies are in progress to identify these proteins in the sera of patients with HCC. To our knowledge, this is the first large-scale quantitative proteomic profiling of HCC tissue revealing several novel and known upregulated proteins. Importantly, we were able to identify many of the proteins that have been previously reported to be upregulated in HCC, corroborating earlier findings of differentially expressed proteins. Overall, this study emphasizes that quantitative proteomics technology may help us to identify novel markers for early diagnosis of HCC as well as elucidate molecular mechanisms of HCC pathogenesis.

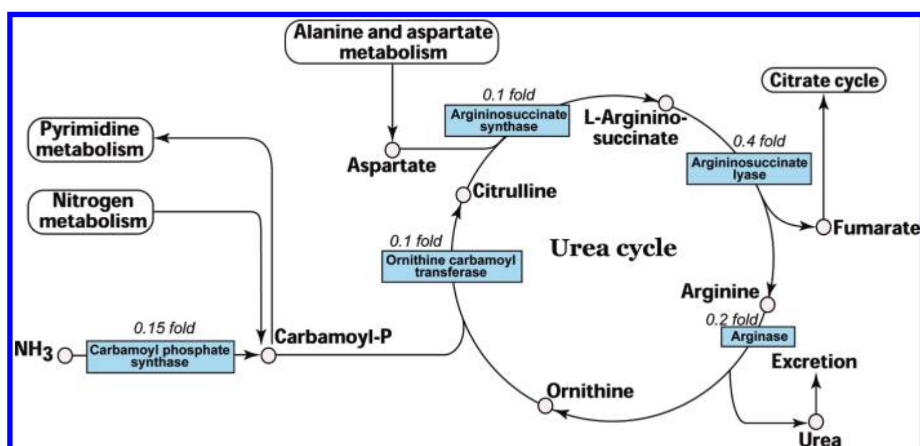

Figure 6. Downregulation of urea cycle enzymes in HCC. The five enzymes of urea cycle were found to be downregulated in the current study. The fold changes observed in iTRAQ experiment are indicated. The urea cycle pathway was redrawn from KEGG database.[34]

Supporting Information Available: Supplemental Table 1. This material is available free of charge via the Internet at http://pubs.acs.org.

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