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Proteomic analysis of HCV cirrhosis and HCV-induced HCC: Identifying biomarkers for monitoring HCV-cirrhotic patients awaiting liver transplantation

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Abstract

Background—Progression from chronic Hepatitis C virus (HCV) infection to cirrhosis and hepatocellular carcinoma (HCC) results in protein changes in the peripheral blood. We evaluated global protein expression in plasma samples of HCV-cirrhotic and HCV-cirrhotic-HCC patients.

Patients and Methods—Plasma samples from 25 HCV-cirrhotic-HCC and 10 HCV-cirrhotic patients were quantitatively evaluated for protein expression. Tryptic peptides were analyzed using Thermo linear ion-trap mass spectrometer (LTQ) coupled with a Surveyor HPLC system (Thermo). SEQUEST and X!Tandem database search algorithms were used for peptide sequence identification. Protein relative quantification was performed using the area under the curve from the select ion chromatogram. A significant fold change between groups was based on controlling the False Discovery Rate (FDR) at less than 5%.

Results—We identified and quantified 2,320 proteins from the analysis of the different protein pattern between HCV-cirrhosis and HCV-HCC samples. Gene ontology terms (GO) classified the more important biologic process related to these proteins as signal transduction, regulation of transcription DNA-dependent, protein amino acid phosphorylation, cell adhesion, transport, and immune response. Seven proteins showed significant expression changes with a FDR<5% between cirrhosis and tumor groups. Moreover, 18 proteins showed significant expression changes (FDR<5%) when plasma samples from HCV-cirrhosis were compared with early HCV-HCC.

Conclusions—Differential protein expression was observed between samples from HCV patients with cirrhosis with and without HCC. Also, differences were observed between early and advanced HCV-HCC samples. This study provides important information for discovery of potential biomarkers for early HCC diagnosis in HCV cirrhotic patients.

Keywords

Hepatitis C virus; Hepatocellular carcinoma; Proteomics; Biomarkers

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Supplemental material: 4 Tables and 2 Figures (This information will be published in our website: http://www.vcuhealth.org/transplant/lab_research.htm)

Introduction

Chronic hepatic disease damages the liver, and the resulting wound-healing process can lead to liver fibrosis and the subsequent development of cirrhosis. One of the leading causes of hepatic fibrosis and cirrhosis is infection with the hepatitis C virus (HCV) (1–4). Of the patients with HCV-induced cirrhosis, 2%–5% develop hepatocellular carcinoma (HCC) (2). The poor survival rate is in part related to the diagnosis of HCC at advanced stages, where effective therapies are lacking (1–4). Surveillance of patients at the highest risk for developing HCC (*i.e.*, patients with cirrhosis) is an important strategy that can potentially decrease the cancer-related mortality rate. Although HCC meets the criteria of a tumor that would benefit from a surveillance program, the poor sensitivity and specificity of currently available tools has prevented widespread implementation of HCC surveillance. Due to its high incidence and poor prognosis when diagnosed at a symptomatic stage, early HCC diagnosis has become a priority.

Progression from chronic infection to cirrhosis and then to HCC usually results in changes in proteins found in hepatic tissues and peripheral blood (5). The most commonly used serum marker of HCC is α -fetoprotein (AFP). However, AFP levels may be normal in up to 40% of patients with HCC, particularly during the early stages (low sensitivity) (6). Furthermore, elevated AFP levels may be seen in patients with cirrhosis or exacerbations of chronic hepatitis (low specificity) (6–8). It has areported sensitivity of 39% to 65% and specificity of 65% to 94% and has multiple limitations when applied to patients with HCV (6–8). In the cohort of patients from the HepatitisC Antiviral Long-term Treatment against Cirrhosis study, 27% of patients with HCV and cirrhosis had an AFP of >20 ng/mL in the absence of HCC, and AFP levels declined with antiviraltherapy (9).

Since 80% of HCC patients in the USA have cirrhosis, optimum care requires the complex analysis of cancer stage to predict recurrence, and the determination of liver reserve to predict suitability of resection *vs.* total hepatic replacement to prevent death from liver failure. The most widely used surveillance test for HCC, ultrasound, is particularly subject to low sensitivity and specificity when applied to cirrhotic patients (10). There is a need to search for more serologic markers which are specifically associated with HCC, especially in the presence of cirrhosis.

Many researchers have identified and described genes that are uniquely up- or down-regulated in HCC tissues by microarray analyses (11–13). Results of these studies provided important information for elucidating the different steps of HCC carcinogenesis. However, these findings might be of limited diagnostic values. First, changes in gene expression levels do not always result in changes in the corresponding protein levels. Moreover, even when changes in gene expression levels result in changes in protein levels, the changes observed in the liver tissue may not correlate well with those in the peripheral blood.

In a recent publication (14), we recognized molecular angiogenic markers differentially expressed between HCC and HCV cirrhotic tissues. Moreover, we were able to identify non-invasive HCC markers from a selected panel of angiogenic proteins. Furthermore, we showed that these markers might be useful for evaluating HCC patients after ablative therapies and/or post-liver transplantation. However, our analysis was limited to a pre-selected small set of angiogenic proteins.

The aim of the present study was to identify plasma protein patterns and differentially expressed single protein markers in patients with HCV cirrhosis and HCV associated hepatocellular carcinoma.

Samples and Methods

Samples

The Institutional Review Board approved the study protocol at VCUHS. We studied 25 plasma samples from HCV-HCC patients and 10 plasma samples from HCV cirrhotic patients. For HCV-HCC patients, histopathological classification was performed according to the Edmondson grading system. Clinical stages were determined according to the American Tumor Study Group modified TNM classification mandated by UNOS (15). Two expert pathologists, with prior knowledge of patient diagnosis, independently performed the pathological classification of the samples.

Sample preparation—High abundant proteins were removed by Genway mixed-12 tip. Resulting flow-through fractions were denatured by 8M urea, reduced by triethylphosphine, alkylated by iodoethanol, and then digested by trypsin as previously described (16).

Mass spectrometry and data analysis—Tryptic peptides (~20 µg) were analyzed using Thermo linear ion-trap mass spectrometer (LTQ) coupled with a Surveyor HPLC system (Thermo). C-18 reverse phase column (i.d.=2.1 mm, length=50mm) was used to separate peptides with a flow rate of 200 µL/min. Peptides were eluted with a gradient from 5 to 45% acetonitrile developed over 120 min and data were collected in the triple-play mode (MS scan, zoom scan, and MS/MS scan). The acquired data was filtered and analyzed by a proprietary algorithm developed by Higgs et al. (17,18). Database searches against the International Protein Index (IPI) human database and the Non-Redundant-homo sapiens database were carried out using both the X!Tandem and SEQUEST algorithms.

Protein identification—The quantified proteins were classified according to identification quality (Priority) (Supplemental Table 1). Priority assignments reflect the level of confidence in the protein identification. Priority 1 proteins have the highest likelihood of correct identification and Priority 4 the lowest likelihood of correct identification. This priority system is based on the quality of the amino acid sequence identification (Peptide ID Confidence) and whether one or more sequences were identified (Multiple Sequences). The Peptide ID Confidence assigns a protein into a 'High' or 'Moderate' classification. This is based on the peptide with the highest peptide ID Confidence (the "best peptide"). Proteins with "best peptide" having a confidence between 90–100% are assigned to the 'High' category. Proteins with "best peptide" having a confidence between 75–89% are assigned to the 'Moderate' category. Proteins with best peptide having a confidence less than 75% are assigned to a 'Low' category and discarded. For priority 1 proteins all peptides with ID Confidence <90% are also discarded. Sequest and Tandem database search algorithms are used for amino acid sequence identification. Each algorithm compares the observed peptide MS/MS spectrum and theoretically derived spectrums from the data base to assign quality scores (Xcorr in Sequest and e-Score in Tandem). These quality scores and other important predictors are combined in a proprietary algorithm that assigns an over all score, %ID Confidence, to each peptide. The assignment was based on a model derived from a random forest recursive partition supervised learning algorithm. The %ID confidence score was calibrated so that approximately X% of the peptides with %ID confidence. X% are correctly identified (18).

Protein quantification—Protein quantification was carried out using the protein quantification algorithm licensed from Eli Lilly and Company (17). Briefly, once the raw files were acquired from the LTQ, all extracted ion chromatograms (XIC) were aligned by retention time. To be used in the protein quantification procedure, each aligned peak must match parent ion, charge state, daughter ions (MS/MS data) and retention time (within a one-minute window). After alignment, area under the curve (AUC) for each individually assigned peak

from each sample was measured, normalized, and these were compared for relative abundance. All peak intensities were transformed to a $\log_2$ scale before quantile normalization (19). Quantile normalization is a method of normalization that essentially ensures that every sample has a peptide intensity histogram of the same scale, location, and shape. This normalization removes trends introduced by sample handling, sample preparation, total protein differences, and changes in instrument sensitivity while running multiple samples. If multiple peptides have the same protein identification, then their quantile normalized $\log_2$ intensities were averaged to obtain $\log_2$ protein intensities. For each protein, an mixed effects analysis of variance (ANOVA) model using the $\log_2$ quantile normalized protein intensity (y) as the dependent variable was fit as follows:

$$y_{ijk} = \mu + \tau_i + \alpha_{j(i)} + \varepsilon_{i(jk)}$$

where μ represents the overall mean, τ represents the fixed effect for the i^{th} group (e.g., cirrhosis or HCC), α represents the random effect due to the j^{th} sample within group i and ε the random error term for the k^{th} replicate of sample j within group i .

All of the injections were randomized and the instrument was operated by the same operator for this study. Fold-changes were calculated as $\text{sign}(T-C) \cdot 2^{|T-C|}$ where T and C are the group means on a $\log_2$ scale. The p -value threshold was set to control the False Discovery Rate (FDR) at 5%.

Spiked Constant Internal Standard—For testing the assay, chicken lysozyme was spiked at a constant amount of total protein before tryptic digestion.

Ingenuity pathway analysis—Proteins identified as being differentially expressed were subsequently analyzed using the Ingenuity pathway analysis software. The Ingenuity pathway analysis program (<https://analysis.ingenuity.com>) uses a knowledge base derived from the literature to relate gene products, based on their interaction and function. This software is designed to identify dynamically generated biological networks, global canonical pathways and global functions. Basically, the identified proteins with their fold change and corresponding Swiss-Prot accession numbers are uploaded as an Excel spreadsheet file into the Ingenuity software. Ingenuity then uses these data to navigate the Ingenuity Pathways Knowledge Base and extract an overlapping network(s) between the candidate proteins. A score better than 2 is usually attributed to a valid network (the score represent the log probability that this network was round by random chance).

Results

Samples

The HCC stages for the studied HCV-HCC patients at the on-study time included: 5 T1N0M0, 7 T2N0M0, 6 T3N0M0, 4 T4N0M0, and 3 T4N0M1. For HCV-cirrhotic patients, liver histology evaluation was performed using Knodell score and Ishak grade (20). Characteristics of the studied patients are shown in the Supplemental Table 2. The analysis of the AFP protein values between plasma samples from HCV-cirrhotic vs. HCV-HCC patients was not statistical significant (30.3 ± 50.5 ng/mL vs. 73.1 ± 83.4 ng/mL, $P=0.33$)

Quality control: Spiked Constant Internal Standard (SCIS)

No significant changes were observed between groups for the SCIS. The Figure-1 shows the Sechart and VARchart for chicken lysozyme. The maximum fold change among HCV-cirrhosis and HCV-cirrhosis-HCC is displayed.

Protein identification

A) Differential protein expression patterns between HCV-cirrhosis and HCV-cirrhosis-HCCs: Analysis of protein expression between plasma samples from HCV-cirrhotic patients with and without HCC was performed. In this study, a total of 2,320 proteins were identified and quantified (summarized in Table 1A). Proteins were categorized into priority groups based on the quality of the protein identification. SEQUEST and X!Tandem database search algorithms were used for peptide sequence identification. Each algorithm compared the observed peptide MS/MS spectrum and a theoretically derived spectra from the database to assign quality scores (*XCorr* in SEQUEST and *E-Score* in X!Tandem).

As it is shown in the Table 1A, two hundred and fifty six proteins with a protein priority of 1 were identified with 6 proteins showing significant fold change in its expression between the studied groups. Five hundred and fifteen protein priorities of 2 were identified while just one protein showed a statistically significant fold change. A volcano plot the q-Value (estimate of the FDR) on the vertical axis versus fold change on the horizontal axis is shown (Figure-2) to visualize the relationship between significance (q-Value <0.05) and size of biological change. The protein intensities for each sample and every protein was filtered to include only high quality proteins identified, defined as those with protein priority of 1 or 2. Thereafter, the dimensionality of the dataset was reduced by including only those proteins having a variance greater than the mean variance among all high quality proteins. Thereafter, hierarchical clustering using Euclidean distance on the quantile log₂ normalized intensities and Ward's method was performed and the resulting heatmap and dendrograms were displayed (Figure-3).

Gene Ontology (GO) annotations classified the function of the differentially expressed protein as principally related with protein binding (242 proteins), ATP binding (49 proteins), DNA binding (33 proteins), calcium ion binding (32 proteins), and transcription factor activity (30 molecules). Signal transduction, regulation of transcription, DNA-dependent; protein amino acid phosphorylation; cell adhesion; and G-protein coupled receptor protein signaling were the more important biological processes associated with the differentially expressed proteins.

The proteins with statistically significant fold changes (q-value<0.05) included Properdin precursor (IPI00021364.1), Apolipoprotein D precursor (IPI00006662.1), A Chain A, Human Serum Transferrin (7245523), Apolipoprotein C-III precursor (IPI00021857.1), 13 kDa protein (PI00657670.1), Platelet basic protein precursor (IPI00022445.1), and Isoform 2 of Heterogeneous nuclear ribonucleoprotein A/B (IPI00334587.1). The rank 1 protein plot is shown as an example in the Figure-4. Rank was assigned by sorting all the proteins in the following order: Significant Change (Yes, No), Priority (1–4) and significance (measured by the smallest q-Value the estimate of the FDR).

When a FDR <10% was used, 40 proteins were identified as statistically significant differentially expressed between groups (Supplemental Table 3).

B) Differential protein expression patterns between HCV-cirrhosis and HCV-cirrhosis-early HCCs: Analysis of protein expression between plasma samples from HCV-cirrhotic patients and patients with early HCV-HCCs (T1N0M0 and T2N0M0) was also performed (Table 1B). From this analysis we observed that 18 proteins showed differential expression levels between groups (q-Value<0.05). The proteins are shown in the Table 2. The levels of twelve of these proteins were increased in plasma samples of HCV-HCC patients with early HCCs including Complement C1r subcomponent (IPI00296165.5), Complement subcomponent 7 (899271), Galectin-4 (IPI00009750.1), and Interleukin-27 (IPI00302598.3), among others. Among the under expressed proteins in early HCV-HCC plasma samples, we observed Human Serum Transferrin (Chain A) (7245523) and precursors of Apolipoprotein C-I (IPI00021855.1) and C-II (IPI00021856.3). Thirty eight proteins were differentially

expressed between HCV-cirrhosis and early-HCV-HCC plasma samples when a FDR <10% was used.

To assess the diagnostic utility of the identified proteins, we included the log2 quantile normalized expression values for the 10 significant proteins with priority 1 from Table 2 and included them in a multivariable logistic regression model derived to predict class (cirrhosis vs. tumor). Using a forward variable selection strategy, the final model included proteins Apolipoprotein C-III precursor (IPI00021857.1) ($P=0.033$) and Human Serum Transferrin (Chain A) (7245523) ($P=0.015$). The area under the Receiver Operating Characteristic (ROC) curve for this model is 0.93, indicating good accuracy (Supplemental Figure-1).

C) Differential protein expression patterns between early and advanced HCV-HCC

plasma samples: When plasma samples from patients with early-HCV-HCC were compared with plasma samples from patients with advanced HCV-HCC (T3N0M0, T3N0M0, and T4N0M1) 12 proteins were statistically differentially expressed (FDR <5%) (Supplemental Table 4). Four of these proteins, Solute carrier family 25, member 26 (IPI00465034.2), Isoform 1 of Cartilage acidic protein 1 precursor (IPI00451624.1), Interferon regulatory factor 6 (IPI00024290.1), CDC45 related protein (IPI00025695.1) were only differentially expressed for this specific group comparison analysis.

Network analysis: The networks and functional analyses were generated through the use of Ingenuity Pathways Analysis (Ingenuity Systems®, www.ingenuity.com). The 2,320 proteins that were identified and quantified in the study were overlaid onto a global molecular network developed from information contained in the Ingenuity knowledge base. Networks of these focus proteins were then algorithmically generated based on their connectivity. Fifteen networks were identified in the protein list with 6 networks with a score higher than 15 and with at least 10 molecules. The more important associated network functions included immunological diseases, cell death, cancer, and tumor morphology. The top scored network (score=56, molecules=26) is shown in the Supplemental Figure-2. To analyze the overlapping network function between proteins whose expression were found to be altered when plasma samples from HCV cirrhotic patients were compared with samples from HCV-HCC patients, we uploaded the 45 proteins that were identified as differentially expressed (FDR<10%) with their accession numbers and fold difference in the expression level into the Ingenuity pathway analysis software. Just one overlapping network was proposed by the software with a score of 14 (score .2 is significant; it represents the log of the probability that the network was found by chance). The most relevant functions extracted from this network were related to cell signaling, molecular transport, small molecule biochemistry, cell cycle, and cell morphology. The top canonical pathways associated with this network were acute phase response signaling ($P=0.002$) and complement system ($p<0.0001$).

From the analysis of the 42 differentially expressed proteins (FDR <10%) between HCV-cirrhosis and early HCV-HCC plasma samples, one network (score=9) was also identified. The most relevant functions extracted from these networks were related to cell signaling, cell-to-cell signaling and interaction, cell death, cellular compromise, and cell morphology.

Evaluation of transferrin levels in blood samples: Blood samples from 25 patients (including both cirrhotic HCV and HCV-HCC patients) were collected at the same time that those samples used for proteomic analysis and evaluated for the iron panel (iron, transferrin, and transferrin saturation) as a part of the clinical evaluation of the patients. The transferrin values (expressed in mg/dL) obtained using a Turbidimetric immunoassay (TIA) were compared with the results from the protein quantification using Thermo linear ion-trap mass spectrometer (LTQ) coupled with a Surveyor HPLC system (Thermo). There was a significant correlation between the two methods (Spearman's correlation=0.46, $P=0.02$).

Discussion

The low efficiency of current HCC therapy urges the identification of tumor-specific markers as potential therapeutic targets, and for the early detection of the disease. As consequence of the strong association between HCV infection, chronic liver disease, and progression to HCC, this high risk group population can be monitored on a regular basis to detect early cancerous lesions. Detection and diagnosis of HCC at an early stage may significantly improve the survival of patients. Currently used methods for detection of liver tumors, however, rely largely on radiographic imaging techniques that are not practical for population-based screening. Thus, considerable effort has been expended toward identification of practical approaches for noninvasive detection of HCC. The ideal biomarker for this type of application is one that can be detected with good sensitivity in a biological sample from the patient in a noninvasive manner (e.g., blood, urine). For HCC, blood represents the best source for detection of cancer-related biomarkers. However, despite an increasing number of noninvasive tests and imaging techniques, detection of liver cirrhosis and hepatocellular cancer is often difficult in chronic hepatitis C infected patients (6–8).

Recent technological advances in transcriptomics and proteomics make it possible to examine expression profiles at the mRNA and protein level. Such approaches are expected to establish the molecular definition of the non-tumor and cancer states of each patient and contribute to the discovery of diagnostic markers and therapeutic targets.

The present study therefore aimed at the identification of plasma protein patterns and single protein markers to differentiate HCV-liver cirrhosis and HCV-hepatocellular carcinoma for monitoring high risk HCV-cirrhotic patients awaiting liver transplantation.

Studying the protein profiles in plasma samples of HCV-cirrhotic patients with and without HCC (HCV-HCC samples included different TNM stages), we were able to identify a panel of differentially expressed proteins. Specifically, when plasma samples from HCV-cirrhotic patients were compared to HCV-HCC samples, seven proteins were significantly differentially expressed between groups ($q\text{-Value} < 0.05$).

Precursors of Apolipoprotein D, Apolipoprotein C-III, Apolipoprotein C-IV, Apolipoprotein C-I, and Apolipoprotein C-II were identified under expressed in HCV-HCC samples when compared with HCV-cirrhosis ($\text{FDR} < 10\%$). Most plasma apolipoproteins, endogenous lipids and lipoproteins are synthesized in the liver (21,22), which depends on the integrity of cellular functions of liver (22,23). Under normal physiological conditions, liver ensures homeostasis of lipid and lipoprotein metabolism (24). Hepatic cellular damage and HCC impairs these processes, leading to alterations in plasma lipid and lipoprotein patterns.

Apolipoprotein C-I has been identified as a marker to differentiate between liver fibrosis and cirrhosis. A role for apolipoproteins in liver fibrosis has been previously published (25). Moreover, apolipoproteins have not only been identified as a serum discriminator of fibrosis but also as a marker in different types of cancer (26). Specifically, apolipoprotein C-I down-regulation was detected to reliably distinguish colorectal cancer patients from healthy controls (27). Thus, besides the function of apolipoprotein C-I in lipid metabolism (28) an additional pathogenic role in liver fibrosis and cancerogenesis appears possible (29).

It was of great interest to evaluate differences in protein expression between HCV-cirrhotic and early HCV-HCC samples. Interestingly, 18 proteins were statistically differentially expressed ($\text{FDR} < 5\%$) between these groups. Galectin-4 and Interleukin-27 were some of the proteins over expressed in the HCV-cirrhotic samples. Galectins are a family of β -galactoside-binding lectins with related amino acid sequences. They are soluble proteins and are generally localized in the cytosol. However, they can accumulate on the cell surface under certain

conditions to play an important role in cell-cell and cell-matrix interactions. Galectin-1 mRNA expression is elevated in fibrosarcoma cells (32) and squamous cell carcinoma (33), whereas Galectin-3 is overexpressed in thyroid tumors (34), colon cancer (35), and squamous cell carcinoma (33). Kondoh et al. (36) reported the association between Galectin-4 and HCC. In contrast to their results in liver, Galectin-4 expression was reported to be down-regulated in colon carcinoma (37), suggesting that the tissue-specific background may be important in determining the functional significance of this molecule for tumor development.

IL-27 is a heterodimeric cytokine that consists of an EBV-transformed gene 3 (EBI3), an IL-12p40-related protein, and p28, a newly discovered IL-12p35-related polypeptide. The former subunit was originally described as a factor secreted by EBI3, while the latter was identified through its homology to the IL-6/IL-12 cytokine family. IL-27 is produced early by activated APCs and induces a rapid clonal expansion of naive but not memory CD4⁺ T cells and synergizes with IL-12 to trigger IFN- γ production in naive CD4⁺ T cells (38). IL-27R consists of WSX-1 (also known as T cell cytokine receptor) and gp130 subunits (39). Researchers have recently demonstrated that IL-27 has a potent ability to induce tumor-specific anti-tumor and protective immunity using colon carcinoma colon 26 (C26) (40,41) and TBJ neuroblastoma (42). Proteins of the complement system (C1r, C7) were also over expressed in cirrhotic HCV samples compared to HCV-HCC while apolipoprotein precursors were down expressed.

Transferrin (Protein ID: 2815575) was also statistically significant down expressed in HCV-HCC plasma samples. The function of this encoded protein is to transport iron from the intestine, reticuloendothelial system, and liver parenchymal cells to all proliferating cells in the body. Perturbations in iron metabolism have been described in HCC (30,31). However, the molecular mechanisms underlying the iron perturbations seen in hepatic malignant cells remain poorly understood. Interestingly, transferrin was one of the proteins identified when a forward variable selection strategy was applied to assess the diagnostic utility of the identified proteins. Moreover, using a second independent method, we were able to correlate the values of transferrin with our proteomic results.

To date, both proteomic and genomic studies have highlighted the heterogeneity of HCC. This heterogeneity emphasizes the complex nature of HCC carcinogenesis and disease progression in which multiple pathogenesis mechanisms seem to be involved. Diagnostic and prognostic molecular markers are being identified by transcriptomic and proteomic analysis of HCC today. However, many of these analyses have been performed on HCC in general, and the studied tissues were HCV infected, HBV infected, infected with both or neither, or the infection status may be unknown (43–45). Because the HCC etiology might have an important effect in the proteomic profiles, our study was limited to samples from HCV-HCC patients.

Our findings provide additional confirmation that a proteomic approach can accurately identify HCC inpatients with cirrhosis. We also showed a different pattern of proteins between HCV-cirrhosis and early HCV-HCC. This might have important implications for its utility in screening for HCC in high risk patients. Evaluations using HCC cell lines might allow functional studies to determine the effects of modulating the expression or activity of the differentially expressed proteins identified in the present study. Based on the identification of differential proteins in HCC cells, these investigations might provide valuable information to recognize changes in cellular pathways that might participate in the development and maintenance of the transformed phenotype (46,47). However, HCC cell lines are relatively homogeneous systems when compared with liver cancer tissue which is composed of multiple cell subpopulations. Further validation studies of large cohorts of patients will be the ultimate step toward clinical use.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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A)

B)

C: HCV-cirrhosis

T: HCV-cirrhosis-HCC

Figure 1.

Spiked constant internal standard. Chicken lysozyme was spiked at a constant amount of total protein before tryptic digestion. Standard error chart (Sechart) (A) and Variability charts for proteins (VARchart) (B) are shown. (A) Sechart: Plot of the group mean protein intensity levels on the log base 2 scale plus or minus standard error. The standard error was computed from the statistical model and is a measure of the precision of the mean. The blue line connects the group means and helps to visualize the treatment trend. (B) VARchart: A plot of the protein intensity levels for each sample on the log base 2 scale. The horizontal purple line is the group mean. Chicken lysozyme was not statistically significant different between groups. The

maximum fold change among two groups comparison is displayed (Rank=181, protein_id=126608, **SigChange=NO**, FoldChange=1.048986)

Figure 2.

Volcano plot. Plot of the q-Value (estimate of the FDR) on the vertical axis versus fold change on the horizontal axis. Priority 1 proteins are blue X's. Plot change is plotted over the -3 to +3.

Figure 3. Heatmap and dendrograms resulting from hierarchical clustering using Euclidean distance quantile log₂ normalized intensities via Ward's method.

A) **B)**

C: HCV-cirrhosis

T: HCV-cirrhosis-HCC

Figure 4.

Standard error chart (Sechart) (A) and Variability charts for proteins (VARchart) (B) are shown. (A) for Properdin precursor protein (Rank=1, Protein ID=IPI00021364.1, Significant Change=YES (q-Value= 0.0427).

Table 1 Overall summary of total identified protein changes between the studied groups

A)							
Protein priority	Peptide ID confidence	Multiple sequences	Number of proteins	Number significant changes	Maximum absolute fold change	Median %CV	
1	High	Yes	256	6	2.5328518	25.68	
2	High	No	515	1	4.3097751	46.29	
3	Moderate	Yes	121	0	1.9842234	34.32	
4	Moderate	No	1428	0	6.6278561	45.55	
Overall			2320	7	6.6278561	42.21	
B)							
Protein priority	Peptide ID confidence	Multiple sequences	Number of proteins	Number significant changes	Maximum absolute fold change	Median % CV	
1	High	Yes	256	14	2.6123492	25.21	
2	High	No	515	10	4.6351169	46.15	
3	Moderate	Yes	121	0	2.1996666	33.97	
4	Moderate	No	1428	2	6.9341747	45.4	
Overall			2320	26	6.9341747	42.03	

Table 2

Differentially expressed proteins between HCV-cirrhotic and early-HCV plasma samples.

Protein ID	Annotation	Best Sequence	%ID Confidence	q-Value
39725934 [*]	Serine (or cysteine) proteinase inhibitor, clade F (alpha-2 antiplasmin, pigment epithelium derived factor), member 1 [Homo_sapiens]	LDLQEINNWWQAQMK	100	0.005576
IPI00296165.5 [*]	Complement C1r subcomponent precursor	ESEQGVYTCTAQGIWK	99.99	0.028806
IPI00021857.1 [*]	Apolipoprotein C- III precursor	SEAEDASLLSFMQGYMK	100	0.028806
IPI00657670.1 [*]	13 kDa protein	SEAEDASLLSFMQGYMK	100	0.028806
899271 [*]	Complement C7	MPYECGPSLDVCAQDER	100	0.028806
IPI00296608.6 [*]	Complement component C7 precursor	MPYECGPSLDVCAQDER	100	0.031994
179716 [*]	Complement protein C7 precursor	MPYECGPSLDVCAQDER	100	0.032936
7245523 [*]	A Chain A, Human Serum Transferrin	MYLGYEYVTAIR	100	0.039341
IPI00021855.1 [*]	Apolipoprotein C-I precursor	MREWFSETFQK	99.9	0.039341
IPI00021856.3 [*]	Apolipoprotein C-II precursor	STAAMSTYTGIFTDQVL SVLK	99.99	0.048941
IPI00023258.5 ^{**}	PREDICTED: similar to Transcription initiation factor TFIIID 105kDa subunit	NKILSLQ	91.43	0.033468
IPI00746871.1 ^{**}	Similar to Alu subfamily SQ sequence	LELLTAR	92.94	0.033468
IPI00009750.1 ^{**}	Galectin-4	LQGGLTAR	95.04	0.033468
IPI00302598.3 ^{**}	Interleukin 27	ELLLLSK	93.05	0.033468
IPI00233524.5 ^{**}	PREDICTED: similar to LETHal family member	LELLGLTG	91.14	0.033468
IPI00305283.1 ^{**}	Ubiquitin ligase SIAH2	LELNGNR	96.46	0.033468
IPI00295368.1 ^{**}	Beta-1,3- galactosyl-O-glycosyl-glycoproteinbeta-1,6-Nacetylglucosamin yl transferase	LEILTVK	96.77	0.033468
IPI00334587.1 ^{**}	Isoform 2 of Heterogeneous nuclear ribonucleoprotein A/B	ESPAGAGTGAAAGAGG ATAAPPSG	90.12	0.044257

^{*} Protein priority score 1^{**} Protein priority score 2