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Identification and Confirmation of biomarkers using an integrated platform for quantitative analysis of glycoproteins and their glycosylations

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Abstract

Hepatocellular carcinoma (HCC) is the most common primary malignant tumor of the liver. However, accurate diagnosis can be difficult as most of the patients who develop this tumor have symptoms similar to those caused by longstanding liver disease. Herein we developed an integrated platform to discover the glycoprotein biomarkers in early HCC. At first, lectin arrays were applied to investigate the differences in glycan structures on serum glycoproteins from HCC and cirrhosis patients. The intensity for AAL and LCA was significantly higher in HCC, indicating an elevation of fucosylation level. Then serum from 10 HCC samples and 10 cirrhosis samples were used to screen the altered fucosylated proteins by a combination of Exactag labeling, lectin extraction and LC-MS/MS. Finally, 27 HCC and 27 cirrhosis serum samples were used for lectin-antibody arrays to confirm the change of these fucosylated proteins. C3, CE, HRG, CD14 and HGF were found to be biomarker candidates for distinguishing early HCC from cirrhosis, with a sensitivity of 72% and specificity of 79%. Our work gives insight to the detection of early HCC, and the application of this comprehensive strategy has the potential to facilitate biomarker discovery on a large scale.

Keywords

Glycoproteins; Hepatocellular; Cancer; Lectin arrays; Biomarkers; Mass Spectrometry

Introduction

Hepatocellular carcinoma (HCC) is the fifth most common tumor and the third most common cause of cancer-related deaths worldwide.[1, 2] According to the World Health Organization, the burden of HCC is expected to continue to increase until 2030, and it is the tumor with the second highest increase in overall death rates in the United States.[3] The 5-year survival rate remains poor for HCC, which is partly related to the lack of accurate diagnosis of HCC at early stages.[4] Curative therapy exists for HCC if diagnosed early, but only about 25% of patients with HCC are diagnosed at an early stage.[5]

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Supporting Information: The total proteins identified from the AAL and LCA fractions are listed in Supplemental Table S1, S2, S3 and S4. The ROC curve and scatter plot of AFP are shown in Supplemental Figure 1. Fluorescent images of AAL-antibody array and LCA-antibody array are shown in Supplemental Figure 2. These materials are available free of charge via the internet at http://pubs.acs.org.

Cirrhosis is the most important risk factor in the development of HCC.[6] Therefore, it is recommended that patients with cirrhosis undergo surveillance with alpha-fetoprotein (AFP) [7] or ultrasound (US).[5] AFP is the most utilized surveillance biomarker for HCC worldwide. Recent systematic reviews of the literature show that the performance characteristics of AFP as a diagnostic and screening test for HCV-related HCC are limited.[8, 9] Another systematic review has shown that the level of evidence for US as a surveillance test is weak (Grade C), and the strength of the data was limited by sample size, variable surveillance frequency, extent of liver disease and verification bias.[10] US as a surveillance test in patients with cirrhosis suffers from a low sensitivity, lack of reproducibility and it is operator dependent.[8] Therefore, there is an urgent requirement for the discovery of a reliable and convenient strategy for the early detection of HCC.

Guidelines on the development of biomarkers for the early detection of cancer have been developed.[11] Of importance in the development of new serum markers for early detection in cancer, is to study patients with early stage tumors. However, the majority of the studies on the development of biomarkers for HCC have been done on patients with more advanced HCC, thereby limiting the value of the markers studied and leading to biased conclusions.[12] For the development of biomarkers for the detection of early stage HCC, it is critical to use only patients with early stage HCC.

Glycoproteins have been found to play a fundamental role in various biological processes, such as cellular regression, immune recognition, protein-protein interaction and intercellular signaling.[13] Protein glycosylation occurs inside the lumen of the endoplasmic reticulum (ER) and the Golgi apparatus, and the modified proteins are then transported into cell surface or secreted to the extracellular matrix. It is proposed that about 50% of human serum proteins are modified in a diversity of glycosylation patterns,[14] the alterations of which have been found to reflect the development and progression of specific diseases, including cancer. Serum glycoproteins have been widely used as therapeutic targets and clinical diagnostic biomarkers such as prostate-specific antigen (PSA) in prostate cancer,[15] CA125 in ovarian cancer,[16] and Her2/neu in breast cancer.[17] In the case of HCC, glycoproteins such as AFP, α-L-fucosidase, glypican-3 and GP73 have been reported to serve as potential serum biomarkers for early detection.[18]

Recently, proteomic technologies such as mass spectrometry and protein microarrays have improved proteomic-based biomarker discovery. Mass spectrometry based approaches have facilitated high-throughput screening of biomarker candidates, many of which are glycoproteins. These approaches include one-dimensional or two-dimensional electrophoresis, [19] label-free quantification,[20] and isotope tag labeling.[21] Alternatively, protein microarrays provide a method to analyze thousands of samples on a large scale with sufficient sensitivity and have been developed as a substitute for ELISA and western blot analysis for the confirmation of these multiplexed biomarker candidates.[22] Lectin based protein microarrays have already been used to identify the change of glycosylation among different stages of cancer, [23] while antibody arrays have been applied to study the change of individual glycoproteins.[24]

In this work, we applied lectin-array methods to investigate the serum glycoprotein profiling differences between cirrhosis and early hepatocellular carcinoma. Selected lectin enrichment was used and the differentially expressed fucosylated proteins were identified using Exactag isotopic labeling and mass spectrometry analysis. The variation of these fucosylated proteins was further confirmed by an AAL-based antibody array. The altered fucosylated proteins discovered by the combination of lectin arrays, comparative proteomics and antibody arrays provide fucosylated glycoproteins as potential biomarkers of early stage hepatocellular carcinoma.

Materials and methods

Sera

Serum samples were obtained from 27 early HCC and 27 cirrohosis patients (Table 1). Twelve of the high abundant proteins from sera were depleted with the IgY-12 LC10 column kit (Beckman Coulter) to remove the 12 most abundant proteins. The depletion was performed with 250 μL of each serum according to the manufacture's procedures. The eluted sample was transferred into a 15mL YM-3 centrifugal device ((Millipore Corp., Bradford, MA), and centrifuged at 4,000 g, followed by washing 3 times with 5 mL deionized water. The sample volume was reduced from 10mL to 500μL. The final protein concentration of each serum sample was measured using the protein assay kit (Bio-rad).

Lectin array

A lectin array was produced using sixteen lectins (Figure 1a) which were dissolved in 10% PBS to a concentration of 1 mg/ml and spotted on 16 pad nitrocellulose slides (Avid, Grace Bio-Labs) using a piezoelectric noncontact printer (Nano plotter; GESIM). The total volume of each spot was 2.5 nL, which results from spotting of 500 pL for 5 times. Each lectin was printed in triplicate. The slides were incubated in a humidity-controlled incubator (> 45% humidity) overnight to allow lectin immobilization. After incubation, the slides were blocked with 1% BSA/PBS for 1 h and washed three times with PBST (0.1% Tween 20 in PBS).

Five micrograms of protein from each depleted serum sample were labeled with EZ-link iodoacetyl-LC-biotin (Pierce) for 2 h then the reaction was stopped by adding 1 μL 2-mercaptoethanol (Sigma). The labeled sample was diluted by 100 times followed by incubation with each block on the slides for 1 h. After washing with PBST for 5 min, the slides were incubated with streptavidinylated fluorescent dye (Alexa555; Invitrogen Biotechnology) for 1 h so that the reaction between the streptavidin and the biotin group of the serum proteins which bind to different lectins can proceed. The intensity from each spot was detected using fluorescent detection with a microarray scanner (Genepix 4000A; Axon).

Exactag Labeling

One hundred micrograms of proteins from each depleted serum were labeled with one of the ExacTag (PerkinElmer) reagents which labels the cysteine residues to generate different isobaric tags, followed by mixing the ten labeled samples (5 of HCC group and 5 of cirrhosis group). Then the pooled sample buffer was exchanged to lectin binding buffer (1×PBS, pH 7.4, 1mM MgCl2, 1mM MnCl2) using Ultracel YM-3. Both forward labeling (cirrhosis labeled with 5 light isobaric tags and HCC labeled with heavy tags) and reverse labeling (cirrhosis labeled with 5 heavy isobaric tags and HCC labeled with light tags) was performed independently on different samples to eliminate the bias generated from the labeling procedure.

Lectin Extraction of Fucosylated Proteins

Agarose-bound Aleuria aurantia lectin (AAL) and agarose-bound Lens culinaris agglutinin (LCA) were purchased from Vector Laboratories (Burlingame, CA). The extraction of fucosylated proteins was performed essentially as described previously[25] with some modifications. Briefly the column packed with 1 mL agarose-bound AAL or agarose-bound LCA was first washed with 3 mL of binding buffer. Two hundred micrograms of Exactag labeled proteins in 1 mL of binding buffer were loaded onto the column and incubated for 15 min. The column was washed with 5 volumes of binding buffer then the captured glycoproteins were released with 4 volumes of elution buffer (300 mM fucose or 200 mM α-methyl glucoside and 200mM α-methyl mannoside in binding buffer). The sample was concentrated using Microcon YM-3 to 200 μL in 25 mM NH_4HCO_3.

Mass Spectrometry

The fucosylated glycoproteins were digested with trypsin at 37 °C overnight. The resulting peptides were analyzed by LC-MS/MS using an LTQ mass spectrometer (Thermo Finnigan, San Jose, CA). Chromatographic separation of peptides was performed on a Paradigm MG4 micropump system (Michrom Biosciences Inc., Auburn, CA) equipped with a C18 separation column (0.1 mm × 150 mm, C18 AQ particles, 5 μm, 120 Å, Michrom Biosciences Inc., Auburn, CA). Peptides were separated with a linear gradient of acetonitrile/water containing 0.1% formic acid at a flow rate of 300 nl/min. A 120 min linear gradient separation was used. The MS instrument was operated in positive ion mode. The ESI spray voltage was set at 2.5 KV, and the capillary voltage at 30 V. The ion activation was achieved by utilizing helium at a normalized collision energy of 35%. The data were acquired in data-dependent mode using the Xcaliber software. For each cycle of one full mass scan (range of m/z 400–2000), the three most intense ions in the spectrum were selected for tandem MS analysis, unless they appeared in the dynamic or mass exclusion lists.

Data Analysis

All MS/MS spectra were searched against the IPI database (IPI.human.v3.39). The search was performed using SEQUEST algorithm version 27 incorporated in Bioworks software version 3.1 SR1 (Thermo Finnigan). The search parameters were as follows: (1) Fixed modification, Carbamidomethyl of C; (2) variable modification, oxidation of M; (3) allowing two missed cleavages; (4) peptide ion mass tolerance 1.50 Da; (5) fragment ion mass tolerance 0.0 Da; (6) peptide charges +1, +2, and +3. The identified peptides were processed by the Trans-Proteomic Pipeline (TPP) [26]. This software includes both the PeptideProphet and ProteinProphet programs. The database search results were first confirmed using the PeptideProphet software, and then the peptides were assigned for protein identification using the ProteinProphet software. In this study, both the PeptideProphet probability score and the ProteinProphet probability score were set to be higher than 0.9. This resulted in an overall false positive rate below 1% [27].

The Exactag Analysis software 3.0 (PerkinElmer) was applied to quantitatively assess the relative peak intensity of featured isobaric tags to analyze the protein variation between the HCC and cirrhosis.

AAL- Antibody Array

Antibody array was performed essentially as Li et al.[28] described with some modifications. Twenty-four antibodies used in this study were listed in Supplemental Table S5. Each antibody was diluted to a 50 μg/μL. The printed slides were blocked with 1% BSA in PBST (0.1% Tween20 in PBS) for 1 h after being oxidized by $NaIO_4$ and derivatized with MPBH and Cys-Gly (Sigma). Serum samples were diluted at 1:400 with 0.1% Brij in PBST, then 100 μL of each diluted serum was applied to each block which was separated by a SIMplex 16 Multi-Array device (Gentel) at random (Figure 2a). After 1 h incubation, the slides were rinsed with PBST thrice then incubated 1 h with 10 μg/μL biotinylated AAL (Vector Laboratory). The response from each spot was detected by a microarray scanner after 1 h reaction with Streptavidinylated fluorescent dye. Two independent experiments were performed with the 54 samples.

Microarray Data Acquisition and Analysis

Genepix Pro 6.0 was used to extract the numerical data from each spot on the slides. A threshold of signal-to-background ratio was set at 3. The background subtracted median intensity of each spot was taken as a single data point into analysis. The triplicate arrays were averaged after standardization. The Student-T test was applied to investigate the differences between the

glycosylation or glycoprotein expression in HCC and cirrhosis. All the statistical analyses were performed using SPSS 13.0.

Western Blotting

Western blotting was performed as described previously [21]. Antibodies used include: mouse anti-Complement C3, mouse anti-Ceruloplasmin, mouse anti- Histidine rich glycoprotein, rabbit anti-CD14, rabbit anti-Hepatocyte growth factor, rabbit anti-Vitamin D binding protein, and rabbit anti-Afamin. All the antibodies were purchased from Abcam.

Results

Lectin Array

A lectin-array consisting of 16 selected lectins was used to investigate which glycan structures showed the largest changes between HCC and cirrhosis. The difference for AAL was 14.8% whereas for LCA the difference was 37.0% between early HCC and cirrhosis (Figures 2b and 2c). AAL and LCA have similar binding specificity to fucosylated N-linked glycans. AAL recognizes α1-3/α1-4 and α1-6 fucose while LCA specifically recognizes core fucose of N-glycans.

Protein Identification and Quantitation

Based on the lectin-array results, AAL and LCA were chosen for extraction of the fucosylated glycoproteins from the Exactag labeled proteins. Proteins identified from both forward and reverse labeling were further quantitatively analyzed. A total of 98 proteins were identified and quantified from the AAL extracted fraction and 56 proteins were identified from the LCA extracted fraction (Supplemental Table 1, 2, 3&4). A representive base peak chromatogram and a MS/MS spectrum of a peptide from Histidine-rich glycoproteins are shown in Figure 3.

Exactag Analysis Software 3.0 was used to analyze the intensity of the set of low mass reporters which were generated from the tag-labeled peptides and were unique to each serum sample. The parameters were set as follows: Minimum fit Quality: 1.5Da; Maximum Mass shift: 0.4Da; Minimum Mean Intensity: 50.00; Charge status: +1, +2, +3, +4; Minimum number of Detected Labels: 7 and Minimum Sequence Length: 2. The final intensity of each peptide was normalized based on the total intensity of the sample set. Only those proteins that showed differential expression levels in both experiments ($p<0.05$) were considered to be changed significantly ($p<0.01$) (Table 2&3). Complement C3, histidine-rich glycoprotein and CD14 were found to be significantly increased in the serum of the HCC group from both AAL and LCA enriched fractions.

Antibody Array

To confirm the protein variation detected by the Exactag labeling method, the AAL-overlay antibody array was used to achieve the expression analysis of a particular fucosylated glycoprotein from the original serum sample without depletion. Twenty six serum proteins were selected based on the proteins determined using Exactag Labeling in the present study and also other cancer biomarker studies. A representative image of antibody arrays from one HCC serum and one cirrhosis serum is shown in Figure 4a. Fifty-four arrays with 27 serum samples from HCC patients and 27 serum samples from cirrhosis patients were analyzed using the background subtracted mean intensity from each antibody (Figure 4a). A linear regression analysis of histidine-rich glycoprotein response to AAL was conducted from two independent antibody array experiments with the same sample set (Figure 4b). The Pearson correlation coefficient was 0.76.

The student's t-test was applied to analyze the variance of protein response to AAL in HCC and cirrhosis serum samples. The arrays showed that the Exactag labeling results, complement C3, ceruloplasmin, histidine-rich glycoprotein, CD14, and hepatocyte growth factor showed significantly higher response in HCC sera than in the cirrhosis sera ($p<0.05$, Figure 4b). However, angiotensinogen, which was detected as increased in the HCC sera by Exactag labeling, showed no significant difference between HCC and cirrhosis in the antibody array test.

The ROC curves in Figure 4c were constructed for each of the 5 fucosylated proteins that showed differential expression to distinguish early hepatocellular carcinoma from cirrhosis. The Area under the ROC curve (AUROC) for complement C3, ceruloplasmin, histidine-rich glycoprotein, CD14, and hepatocyte growth factor was 0.737, 0.733, 0.750, 0.676, and 0.641. The combination of the 5 proteins had an AUROC of 0.811, with specificity of 72% at a fixed sensitivity of 79%, while AFP has an AUROC of 0.661, with specificity of 35% at a fixed sensitivity of 79% (Supplemental Figure S1). The ROC curve of patients with HCV etiology was also performed to test the significance of the HCV status in the results, however, little change was observed (Supplemental Figure S3).

Principal component analysis (PCA) was also performed on the total data from the antibody array and data from the 5 proteins with significantly increased response in HCC, to provide the partial visualization of the relationships among the samples in a reduced dimension plot (Figure 5d), which revealed that these proteins can be used to distinguish between HCC and cirrhosis serum samples.

Western blotting

Western blotting was employed to further confirm the variation of these fucosylated proteins including C3, CE, HRG, CD14 and HGF. As shown in Figure 6, all of the five proteins were detected at higher levels in the HCC serum than those in cirrhosis serum. As control proteins, both VDB and AFM showed almost the same expression levels between HCC and cirrhosis.

Discussion

Alteration of glycoproteins has often been related to tumor progression and provides a useful clue for serological biomarker development. In this study, we used lectin arrays to evaluate the difference of glycosylation type of depleted serum between early HCC and cirrhosis patients. Our results suggest that the level of protein fucosylation was strongly increased in early HCC serum while other kinds of glycosylation showed no significant variation. Hyper-expression of specific fucosylated glycoproteins have been reported in a number of malignant tumors including HCC,[29] while our lectin array results suggest not only an individual fucosylated protein upregulated but also the fucosylation levels of all the serum proteins (after depletion) can serve as a potential biomarker to distinguish between early HCC and cirrhosis. It has been reported that fucosyltransferases and GDP-fucose transporter involved in the fucosylation process were overexpressed in HCC patients,[30] and Comunale et al. and other groups have found increased levels of core fucosylation in HCC via glycan analysis of total serum,[31, 32] This is consistent with the higher level of total fucosylated proteins in HCC serum detected by the lectin array in the present work.

We further utilized Exactag Labeling and mass spectrometry based detection to investigate which fucosylated proteins contribute to the alteration of fucosylation levels in HCC serum. Exactag reagents were designed to label the thiol group at the protein level[33] which allowed us to proceed with the lectin extraction procedure[34] with the pooled samples as shown in the workflow of Figure 1. Using such a method we have identified several fucosylated glycoproteins (Table 2 and Table 3) which were expressed more highly in HCC serum than

cirrhosis. The variance of these proteins were detected from depleted serum followed by the lectin extraction procedure, so that we also applied the AAL-antibody array to test if these differences still exist in the original serum. Antibody arrays have been used to capture a specific protein from serum to study the changes in its glycan structure, with a high level of specificity and reproducibility. However, the method requires a discovery platform to select which antibodies should be used for screening. In this work we used the Exactag labeling and antibody array together as an integrated strategy for biomarker discovery and confirmation in a high-throughput manner. Consistent with the Exactag based results, C3, CE, HRG, CD14 and HGF were also found to be increased in the HCC serum. The combination of these proteins had an AUROC of 0.811, with specificity of 45% at a fixed sensitivity of 95%, which is better than AFP for the detection of early HCC (Supplemental Figure S2).

It has been reported by Comunale et al. and Wang et al. that Hemopexin was increased in the serum from HCC patients.[31, 35] Comunale et al. also found the combination of GP73, Fc-kin and AFP could be used as a panel to distinguish the HCC from cirrhosis, by blocking the α-gal antibodies before analysis of specific proteins.[36] Their studies are not discovery but rather confirmation of several glycoproteins, using HCC patients with different stages. In our study, we included 27 patients with very early HCC to determine whether there are differences in the glycoprotein profile. MELD score shows that they are normal indicating normal hepatic synthetic function (Table 1). Glycoproteins differentially expressed in very small tumors are more likely to be clinically meaningful biomarkers in future studies. We did not observe significant changes in either hemopexin or kininogen with our early stage samples, indicating the fucosylation level of a specific protein may alter with the development of HCC.

Lectin-antibody arrays were first developed to profile the the glycan variation in multiple samples.[24] Chemical derivatization was employed to block the non-specific binding between lectins and the glycans on the spotted antibodies. Multiple lectins are then used as detection probes to target the glycoproteins captured by antibodies. The blocking of the lectin binding to the spotted antibodies is critical for obtaining accurate results. AAL could be effectively blocked at 50 mM sodium periodate ($NaIO_4$), but in the case of LCA, the non-specific binding to the antibodies can still be detected at 150 mM $NaIO_4$. The same results were observed in our study (Supplemental Figure S2). As for AAL, the average intensity of the arrays incubated with serum was about 20 times stronger than the average intensity of the arrays incubated with PBS after derivatization. In the case of LCA, there was only a 2 times difference due to the high non-specific binding between LCA and antibodies. Therefore, only the AAL-antibody array based confirmation was used in the present work.

The expression levels of Complement C3 (C3), Ceruloplasmin (CE), and Histidine-rich glycoprotein (HRG) have been found to be increased in various tumor processes. CE has been reported to be over-expressed in ovarian cancer and pancreatic cancer,[37, 38] while C3 and HRG have been found to be potential fucosylated biomarkers in the plasma of patients with colorectal cancer, prostate cancer and breast cancer.[39-41] Our results showed that C3, CE and HRG had an AUROC of 0.737, 0.733, and 0.750 in discriminating cirrhosis from HCC, which suggest the change of these proteins may be a common character in cancer caused by the difference between tumor and normal cells. We have also found that hepatocyte growth factor (HRG) and CD14 were hyper-expressed in HCC serum. HRG has been reported to be a critical limiting step in invasive growth of tumor cells in HCC[42], while CD14 has been found to mediate the HRG activation via the CD14/TLR-2 pathway,[43, 44] where the increase of CD14 and HGF may relate to the development of HCC progression.

Conclusion

We have applied a lectin-array method to investigate the greatest change in glycosylation differences in serum from early HCC and cirrhosis. A combined strategy was developed based on Exactag labeling-LC-MS/MS and AAL-antibody array for the identification and confirmation of differentially expressed fucosylated proteins in HCC. Our results suggest that C3, CE, HRG, CD14 and HRG could be used as biomarker candidates to supplement the current diagnostic criteria for HCC. The next step based is to confirm our findings in a larger cohort of patients of diverse gender, ethnicity and etiologies of liver disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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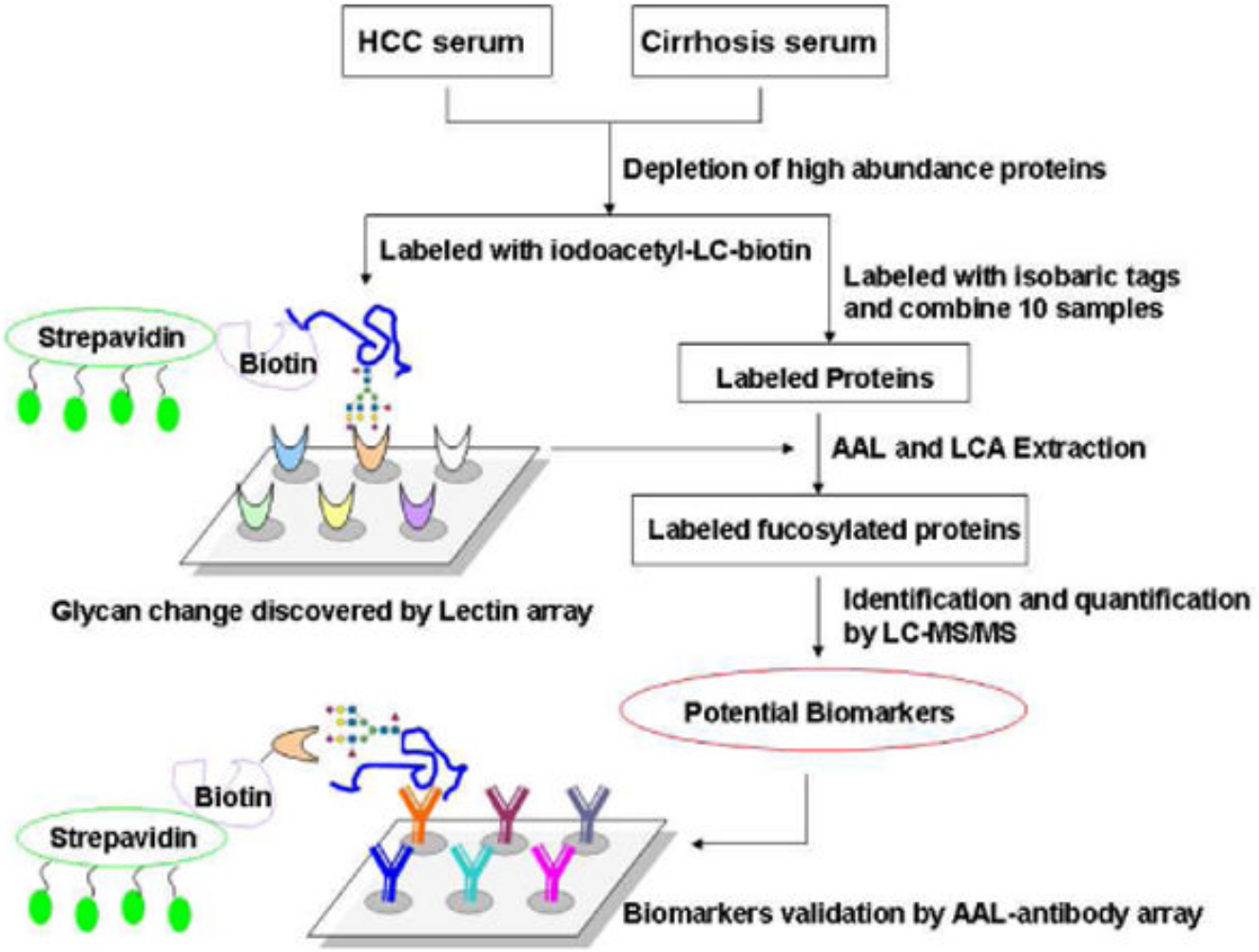

Figure 1.
Workflow showing the integrated strategy for the discovery of serum glycoprotein biomarkers in early hepatocellular carcinoma (HCC).

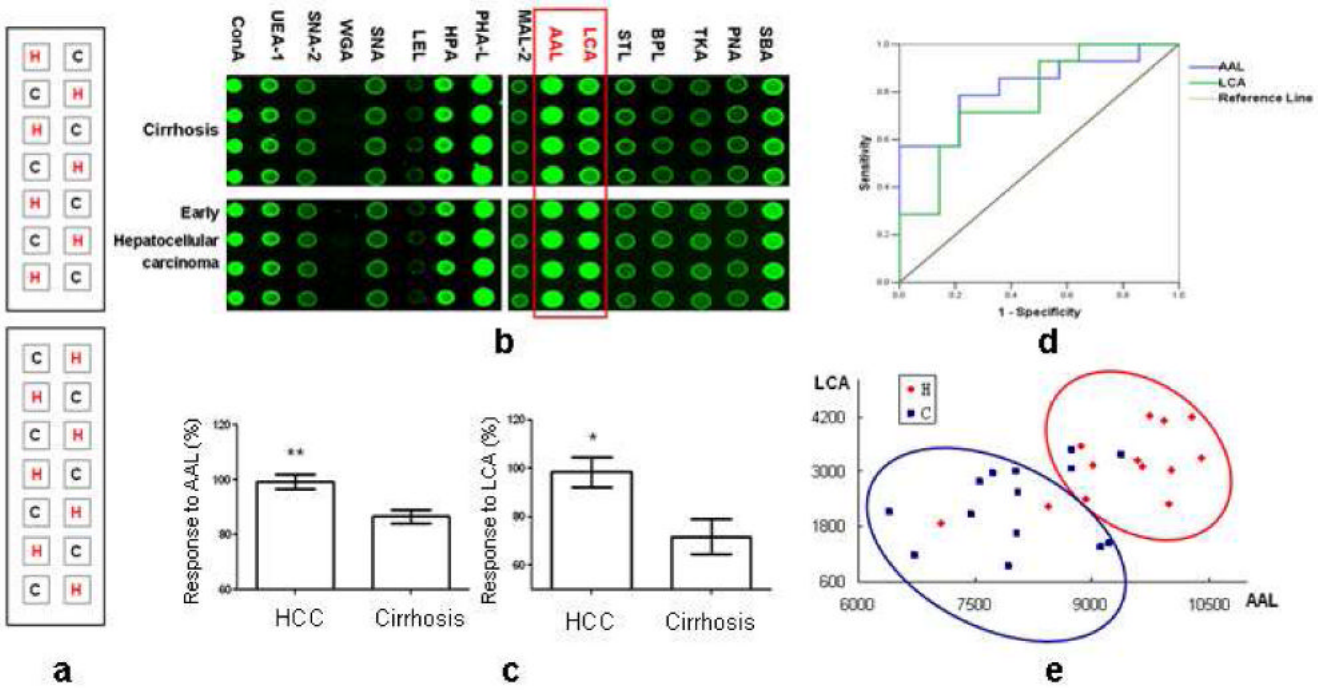

Figure 2.

Lectin Microarray. (a) Sample randomization on the slides. H represents early hepatocellular carcinoma; C represents cirrhosis. (b) Response of 16 different lectins to glycoproteins in depleted serum from HCC and cirrhosis patients. AAL and LCA showed significantly different response to HCC and cirrhosis. (c) Summary of AAL and LCA response. Error bars indicate S.E. from 14 HCC and 14 cirrhosis patients; * $p<0.05$; ** $p<0.01$. (d) ROC curves of sensitivity and specificity for AAL and LCA. (e) Scatter plot of fucosylation level in different serum samples detected by AAL and LCA.

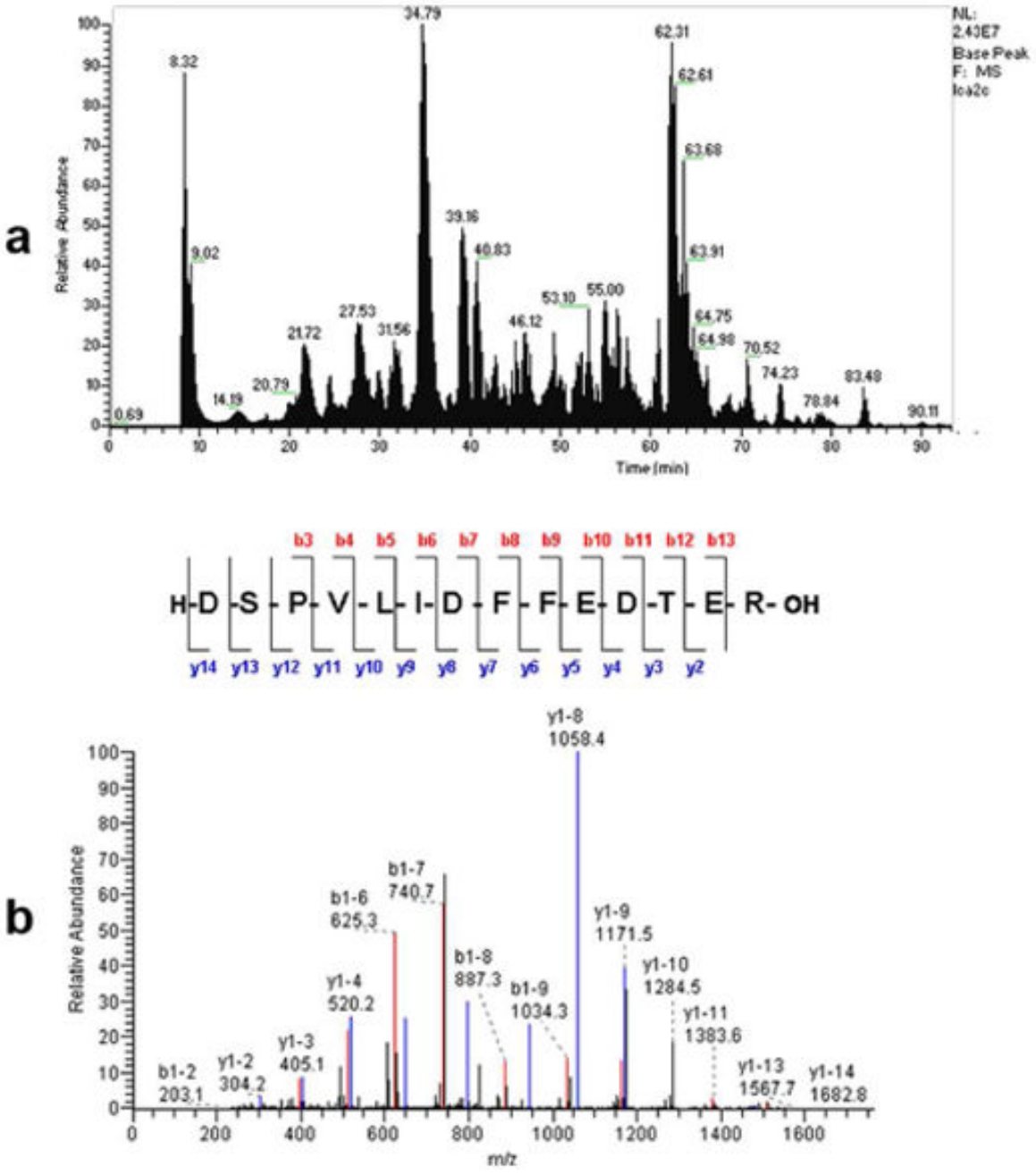

Figure 3.

LC-MS/MS analysis. (a) A representive nano LC-MS/MS base peak chromatogram, showing the detection of peptide ions across 120 min gradient separation (90 min shown here). (b) MS/MS sequencing data of a peptide from histidine-rich glycoprotein identified in the AAL extracted fractions.

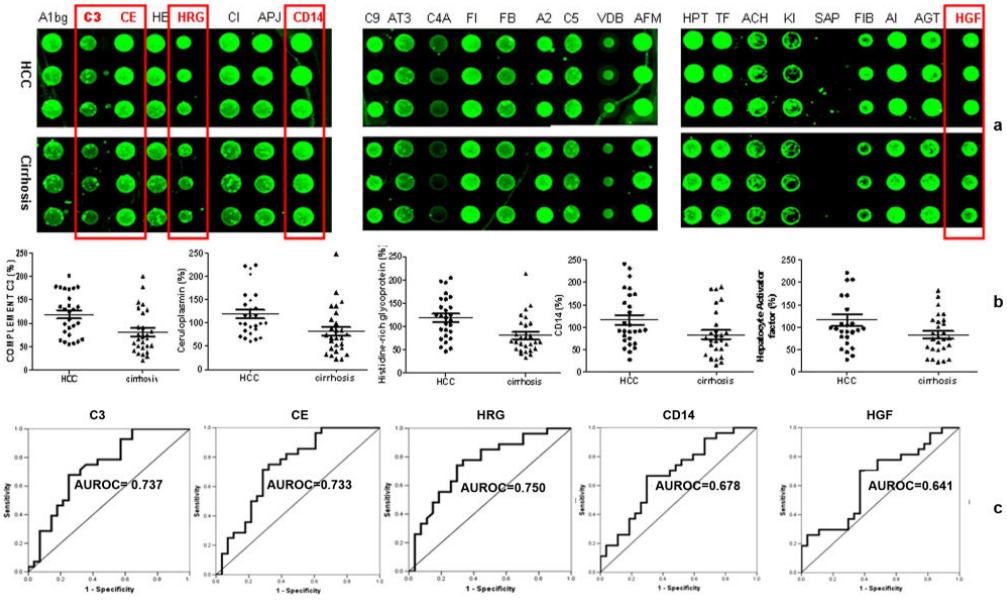

Figure 4.

Fucosylated protein alteration confirmed by Antibody Microarray. (a) AAL-assisted antibody array of 26 selected proteins in the serum from HCC and cirrhosis patients. Proteins which showed significantly different response to AAL between HCC and cirrhosis were indicated by red rectangle ($p<0.05$). (b) Comparison of response intensity of C3, CE, HRG, CD14 and HGF to AAL in HCC and cirrhosis. Each spot represents one serum sample, error bars indicate the standard deviation from 27 HCC and 27 cirrhosis patients. (c) ROC curves for C3, CE, HRG, CD14 and HGF.

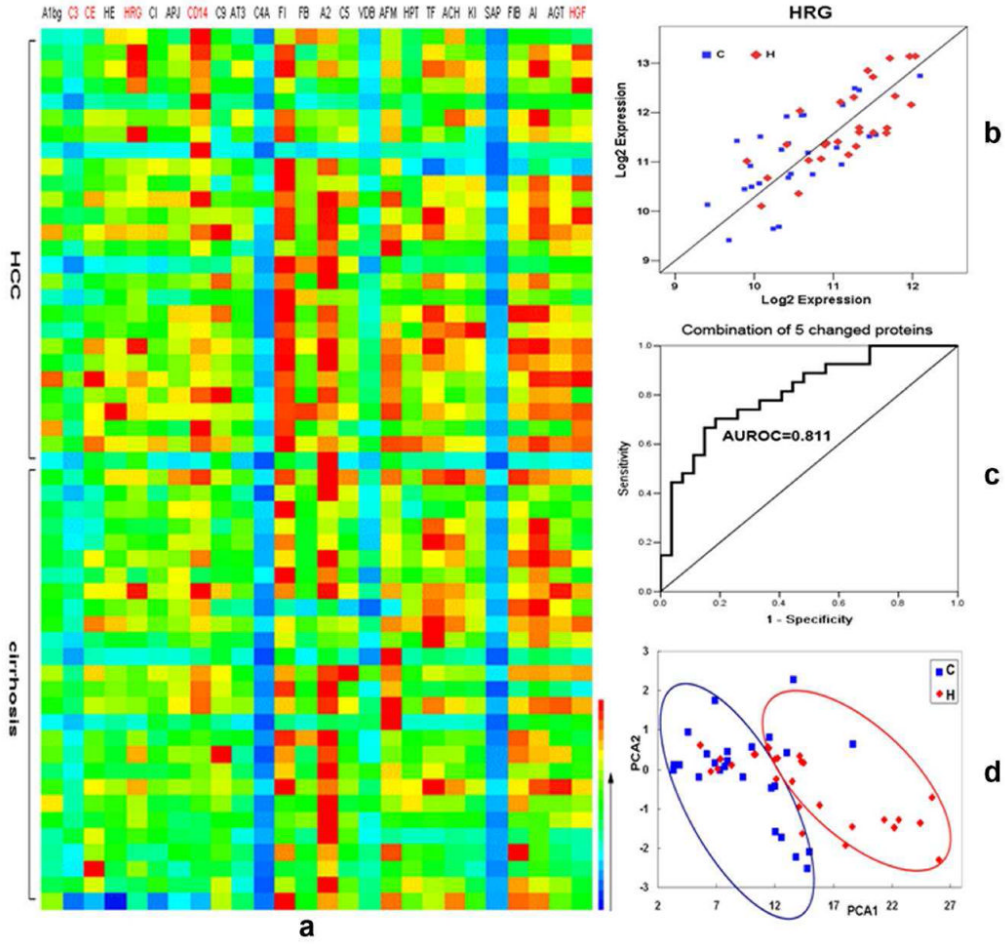

Figure 5.

Statistical analysis of antibody microarray. (a) Heatmap based on the signal intensity of antibody microarray. Each column indicates a selected protein tested in the antibody array experiment, and each row indicates an individual serum sample from HCC or cirrhosis patients. The intensity increased from blue color to red color. (b) Scatter plot in log 2 scale and a regression line of histidine-rich glycorprotein (HRG) response to AAL between every pair of two independent experiments with the same sample set. (c) Combined ROC curve of C3, CE, HRG, CD14 and HGF. (d) Principle component analysis. Shown is the response intensity of 26 selected serum proteins from 27 HCC and 27 cirrhosis patients onto the two leading principal components.

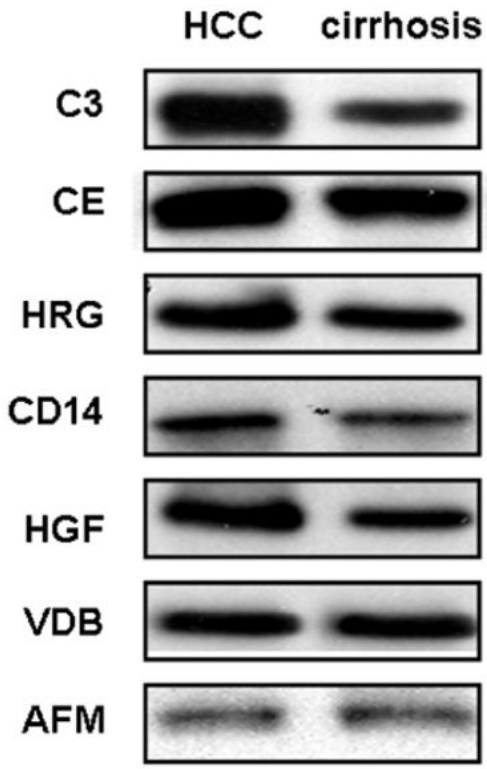

Figure 6.

Western blotting analysis of C3, CE, HRG, CD14, HGF, VDB and AFM. Depleted serum samples from 10 HCC patients (left lane) and 10 cirrhosis patients (right lane) were pooled, respectively. Five micrograms of proteins were separated by a 4%-12.5% gel. The expression level of C3, CE, HRG CD14 and HGF were increased in the serum from HCC, while the control proteins VDB and AFM showed no significant difference between HCC and cirrhosis.

Table 1

Clinical characteristics of 27 patients with HCC and 27 patients with cirrhosis used in biomarker studies.

Disease Diagnosis	Early HCC	Cirrhosis
Number	27	27
Etilogy%(HBV/HCV/ALC/crypto/other)	7/74/7/26/7	7/74/7/26/7
Age	57±12	52±7
Gender M:F%	78/12	59/41
MELD Score	10±4	7±3
AFP Level (ng/nL)	500±1286	9±12
Tumor Size (cm)	1.8±0.2	NA
Number of Lesions	1.2±0.1	NA
Portal Vein Thrombosis (%)	0	NA
Tumor Stage (%) I/II/III/IV	100/0/0/0	

[a] Samples were provided by Division of Gastroenterology, University of Michigan

[b] MELD: Model for end stage of liver disease

[c] AFP level was provided by Division of Gastroenterology, University of Michigan

Table 2

Changed proteins identified from AAL extracted fraction.

Total proteins identified from the AAL enriched fractions are listed in Table S1 and Table S2. Changed proteins that were identified from both AAL fractions and LCA fractions are in bold.

Protein ID	Protein Name	Forward Labeling[a]		Reverse Labeling[b]		*p* value[g]
		Ratio[c]	*p*[d]	Ratio[e]	*p*[f]	
IPI00007199	PROTEIN Z-DEPENDENT PROTEASE INHIBITOR	2.43	0.0006	2.65	0.000216	1.97E-05
IPI00017601	CERULOPLASMIN	2.80	0.0169	1.87	0.022224	1.43E-05
IPI00019580	PLASMINOGEN	4.72	0.0396	1.82	0.034107	3.14E-03
IPI00022371	**HISTIDINE-RICH GLYCOPROTEIN**	2.88	0.0297	2.04	0.009187	1.37E-02
IPI00029193	HEPATOCYTE GROWTH FACTOR ACTIVATOR	3.19	0.0281	1.89	0.044094	5.09E-04
IPI00029260	**MONOCYTE DIFFERENTIATION ANTIGEN CD14**	1.87	0.0122	1.64	0.043387	2.33E-03
IPI00032220	ANGIOTENSINOGEN.	1.87	0.0429	2.11	0.044558	3.26E-03
IPI00736860	PUTATIVE UNCHARACTERIZED PROTEIN ENSP00000374988	2.02	0.0271	2.41	0.006589	1.05E-03
IPI00783987	**COMPLEMENT C3**	1.64	0.0279	1.72	0.032306	2.74E-04

[a] Forward Labeling: Five of cirrhosis samples labeled with 5 light tags and 5 of HCC samples labeled with 5 heavy tags

[b] Reverse Labeling: 5 of HCC samples labeled with 5 light tags and 5 of cirrhosis samples labeled with 5 heavy tags

[c] Protein expression ratios of HCC/cirrhosis detected from forward labeling group

[d] *p*: *p* value for changed proteins in the forward labeling group

[e] Protein expression ratios of HCC/cirrhosis detected from reverse labeling group

[f] *p*: *p* value for changed protein in the reverse labeling group

[g] *p* value: statistical significance of changed proteins between 10 HCC and 10 cirrhosis

Table 3

Changed proteins identified from LCA extracted fraction

Total proteins identified from the LCA enriched fractions were listed in Table S3 and Table S4. Changed proteins that were identified from both AAL fractions and LCA fractions are bold.

Protein ID	Protein Name	Forward Labeling[a]		Reverse Labeling[b]		*p* value[g]
		Ratio[c]	*p*[d]	Ratio[e]	*p*[f]	
IPI00017601	**CERULOPLASMIN**	2.75	0.0398	1.63	0.0056	8.13E-04
IPI00022371	**HISTIDINE-RICH GLYCOPROTEIN**	3.00	0.0416	2.93	0.0135	5.01E-04
IPI00022395	COMPLEMENT COMPONENT C9	2.43	0.0220	3.37	0.0658	1.06E-03
IPI00029260	**MONOCYTE DIFFERENTIATION ANTIGEN CD14**	3.33	0.0415	2.10	0.0001	1.21E-03
IPI00029739	ISOFORM 1 OF COMPLEMENT FACTOR H.	2.39	0.0422	2.09	0.0343	6.07E-04

[a] Forward Labeling: Five of HCC sample labeled with 5 light tags and 5 of cirrhosis sample labeled with 5 heavy tags

[b] Reverse Labeling: 5 of cirrhosis sample labeled with 5 light tags and 5 of HCC sample labeled with 5 heavy tags

[c] Protein expression ratios of HCC/cirrhosis detected from forward labeling group

[d] *p*: *p* value for changed proteins in the forward labeling group

[e] Protein expression ratios of HCC/cirrhosis detected from reverse labeling group

[f] *p*: *p* value for changed protein in the reverse labeling group

[g] *p* value: statistical significance of changed proteins between 10 HCC and 10 cirrhosis