Identification of Candidate Biomarkers in Malignant Ascites from Patients with Hepatocellular Carcinoma by iTRAQ-Based Quantitative Proteomic Analysis

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Almost all the patients with hepatocellular carcinoma (HCC) at advanced stage experience pathological changes of chronic liver cirrhosis, which generally leads to moderate ascites. Recognition of novel biomarkers in malignant ascites could be favorable for establishing a diagnosis for the HCC patients with ascites, and even predicting prognosis, such as risk of distant metastasis. To distinguish the proteomic profiles of malignant ascites in HCC patients from those with nonmalignant liver cirrhosis, an iTRAQ pipeline was built up to analyze the differentially distributed proteins in the malignant ascites from HCC patients (n=10) and benign ascites from hepatic decompensation (HD) controls (n=9). In total, 112 differentially distributed proteins were identified, of which 69 proteins were upregulated and 43 proteins were downregulated (ratio \(<0.667\) or \(>1.3\), respectively) in the malignant ascites. Moreover, 19 upregulated proteins (including keratin 1 protein and rheumatoid factor RF-IP20, ratio \(>1.5\)) and 8 downregulated proteins (including carbonic anhydrase 1, ratio \(<0.667\)) were identified from malignant ascites samples. Functional categories analyses indicated that membrane proteins, ion regulation, and amino acid metabolism are implicated in the formation of HCC malignant ascites. Pathways mapping revealed that glycolysis/gluconeogenesis and complement/coagulation cascades are the mostly affected cell life activities in HCC malignant ascites, suggesting the key factors in these pathways such as Enolase-1 and fibrinogen are potential ascitic fluid based biomarkers for diagnosis and prognosis for HCC.

1. Introduction

Hepatocellular carcinoma (HCC) is the fifth most prevalent cancer in the world and a leading cause of cancer-related death worldwide [1]. Age-adjusted incidence rates among HCC patients have increased faster in recent decades while its mortality is almost equal to its morbidity. Development of HCC is asymptomatic at early stages of the disease, which is attributed to complex causes including chronic liver diseases, hepatitis virus infection, and alcohol abuse. HCC patients rarely can be diagnosed at early stage due to lack of effective biomarkers for the diagnosis. A few blood test based biomarkers like alpha fetal protein (AFP) are used in clinical practice, but the value of these tests for surveillance purposes has not been sufficiently validated. There is an urgent clinical need to identify new HCC biomarkers for improvement of diagnosis and treatment outcome monitoring [2].

Ascites is the pathologic accumulation of fluid in the peritoneal cavity that exceeds certain amount. A majority of HCC are observed in association with liver cirrhosis, while ascites is one of the major complications of liver cirrhosis and indicates poor prognosis [3]. HCC patients generally have malignant ascites which is bloody and opaque compared to benign ascites which is mostly clear. The cell growth of
primary tumor and its metastasis in other parts of body contribute to the formation of malignant ascites, suggesting molecular analysis of malignant ascites from cancer patients may provide valuable information for clinical surveillance, medical screening, and intervention [4]. Ascitic fluid has been analyzed in order to develop a differential diagnosis for malignancy-related cirrhosis, including liver cirrhosis. For instance, one recent study reported that lactoferrin level in ascites is a useful diagnostic tool to identify in cirrhosis patients with spontaneous bacterial peritonitis [5]. Studies of ascitic samples on oncology research were largely focused on breast cancer, and gastric cancer and colorectal cancer, but rarely in HCC. [6–8]. Despite the large amount of information reported about the characterization of ascitic fluid, its diagnostic value in HCC patients is not yet defined.

Other than the widely used genomic analyses such as microarrays and NGS sequencing technologies in molecular biomarker discovery, proteomic-based approaches have shown many advantages in protein biomarkers screening [9–11]. Moreover, protein based studies can assist in systems biology research which could lead to identification of new drug targets [12]. Isobaric tag for relative and absolute quantitation (iTRAQ) is an approach using a multiplexed set of four or eight isobaric reagents to tag protein/peptide and then measure their relative expression levels [13]. This technique allows the protein samples to be pooled after labeling without increasing the complexity of measurement, making it feasible to identify and quantify proteins simultaneously. Compared with other proteomic-based techniques, iTRAQ has been employed to proteomics research on various types of samples including cell lines, tissues, fluids, and even bacteria [14–16]. Hundreds of unique proteins identified by iTRAQ have been predictive in the translational research and clinical practice [17, 18].

In the presented study, an iTRAQ pipeline was built up to analyze the differentially distributed proteins in HCC ascites samples when compared with hepatic decompensation (HD) controls. In total, 19 upregulated proteins and 8 downregulated proteins were identified from ascitic fluid samples (HCC vs. HD). Identified upregulated proteins included keratin 1 and rheumatoid factor RF-IP20 while the downregulated proteins included carbonic anhydrase I. Functional categories analyses showed membrane proteins are key factors in the development of HCC malignant ascites. Pathways mapping revealed pathways of glycolysis/glucogenesis and complement/coagulation cascades are the mostly affected functions involved in HCC malignant ascites, suggesting key factors from these pathways could serve as biomarkers for HCC.

2. Materials and Methods

2.1. Patient Subjects. Patients with primary hepatocellular carcinoma (HCC, n=10) and hepatic decompensation (HD, n=9) were enrolled and their ascitic fluid samples were collected. Characteristics of the enrolled patients are shown (Supplementary Materials, Table S1). The diagnosis for the HCC patients was according to the histological results of liver surgical resections. All protocols related human materials were approved by the ethics committee of Affiliated Cancer Hospital of Guangxi Medical University. Informed consent was obtained and the access to human samples was carried out in accordance with the approved guidelines of the ethics committee.

2.2. Ascitic Fluid Collection. Malignant fluid samples were collected from patients by paracentesis done under sterile condition using 21-gauge needle. The routine testing of ascitic fluid included cell counting and measurement of total protein and albumin. The specific investigations like liver biopsy and ascitic fluid culture were performed as required. Cell debris in the ascitic fluid was removed through centrifugation at 4°C for 20 min at 15,000g. The supernatant was transferred to a sterile tube and the amount of total protein was determined by using a commercial assay reagent kit (Pierce BCA Protein Assay Kit). For the preparation of samples used in iTRAQ assay, 100 μg of each sample was collected carefully in cold sterile PBS containing protease inhibitor cocktail and stored at −70°C immediately. For the subsequent ELISA assay, the total amount of proteins from 10 mL of each ascitic fluid sample was harvested and stored as described above. No additional freeze-thaw was carried out before the detection.

2.3. iTRAQ Proteomics Analysis. The mixed ascitic fluid samples were submitted for iTRAQ analysis (Beijing BangFei Bioscience). Each sample was labeled using iTRAQ Reagent-8plex Multiplex Kit according to the protocol (Applied Biosystems, Foster City, CA). Eight isobaric tags were employed to label the samples from HCC group or HD group. The labeling strategy was established according to the concentration and distribution of total amount protein from all samples measured by BCA protein quantification. The iTRAQ labeling protocol is illustrated (Supplementary Materials, Figure S1). Sample fractionation was carried out by using SCX chromatography column (C18, 3 μm, 0.1 x 2.0 cm polysulfoethyl A column, PolyLC Inc.), as described previously [19]. LC buffers were prepared freshly every day as follows: (1) SCX chromatography buffer A (SCX-A): 20% (vol/vol) acetonitrile and 0.1% (vol/vol) formic acid (pH 2.7); (2) SCX chromatography buffer B (SCX-B): 20% (vol/vol) acetonitrile, 0.1% (vol/vol) formic acid and 1M KCl (pH 2.7). In brief, 100 μl of SCX-A was added to each sample and pooled to a single tube; each tube was further rinsed with 100 μl of SCX-A and added to pooled material. Each entire sample was transferred to a glass sample vial and the volume was adjusted to 1.6 ml with SCX-A and loaded onto the analytical column for a HPLC system (Agilent, Palo Alto, CA) using a 2-ml injection loop and washed with SCX-A at 1 ml min−1 for 40 min. To acquire the separated component, a binary mobile-phase gradient at a total flow rate of 250 μl min−1 was used. The gradient comprised an increase from 0 to 14% SCX-B over 24 min, 14 to 30% SCX-B over 36 min, and 30 to 100% SCX-B in 20 min. The column was then washed with 100% SCX-B for 15 min and reequilibrated with 100% SCX-A for 15 min.

2.4. LC-MS/MS Analysis. The amount of 10 μL loading buffer was added to each sample in a high PH condition to dissolve
the labeled samples before running with Q Exactive HF Orbitrap LC-MS/MS System (Thermo Finnigan). All samples
(each 2.5 μg) were analyzed using an Easy nLC system HPLC
coupled to a Q Exactive mass spectrometer. Peptides were
preconcentrated on a C18 trapping column (3mm × 10 mm
× 20 mm, PolyLC Inc.) for 10 min using 0.1% TFA (v/v) with
a flow rate of 250 μL/min followed by separation. The Q
Exactive HF MS was operated in data-dependent acquisition
(DDA) mode and MS survey scans were acquired from m/z
300 to 1,400 at a resolution of 120,000. Isolation of precursors
was performed by the quadrupole with a window of 1.6 m/z.
The most intense signals were subjected to higher energy
collisional dissociation with a normalized collision energy
(0.054+ m/z + 5) taking into account a dynamic exclusion of
12.0s. Maximum injection times (IT) were 45 ms. Precursor
ions with charge states of +1, +7, +8, or >8 unassigned were
excluded from MS/MS analysis.

2.5. Protein Quantification and ELISA. The MS/MS data
were searched against the Mascot database (uniprot-human_
20151227.fasta) for peptide identification and quantification.
The search result of peptide was filtered by FDR p value
with a cutoff of 0.01. The differentially distributed proteins
were further characterized using the software Proteome
Discoverer 1.4 (Thermo). To further verify the validity of
the iTRAQ-based quantitative proteomic analysis, an enzyme-
linked immunosorbent assay (ELISA) was carried out to
examine and quantify level of carbonic anhydrase I (CA1)
within the ascitic fluid samples, which was detected to be
decreased in the HCC group. The ready-to-use ELISA Kits
were purchased from LifeSpan BioSciences Inc. (catalog No.
LS-F23971) and the experiment was performed in accordance
with the manufacturer's protocol.

2.6. Statistics Analysis. Statistical analysis was performed
using SPSS Statistics 22.0 (IBM). Differences analyses in
protein expression between the HCC and HD groups were
performed using a t-test, and p < 0.05 was taken to indicate
statistical significance. Based on statistical dispersion of the
dataset (a total of 627 proteins were detected), ratio of >1.3
or <0.767 was set as the threshold to identify differentially
expressed proteins, using a cutoff of two times standard
deviation (Supplementary Materials, Figure S2). In addition
to that, ratio of >1.5 or <0.667 was used as a strict
significance cutoff to acquire a short list of the differentially
distributed proteins as indicated in the data legends. Eukaryotic
Orthologous Groups (KOG) and Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes
(KEGG) analyses were considered statistically significant at p
< 0.05.

3. Results

3.1. Differentially Distributed Proteins Identified by iTRAQ
Analysis. To identify HCC-specific protein biomarkers from
HCC ascites proteome, the ascitic fluid samples from HCC
patients and HD control were collected and analyzed by
iTRAQ and MS analysis. Out of the total 627 proteins
detected in the assay, 112 differentially distributed proteins
were identified, of which 69 proteins were upregulated and
43 proteins were downregulated (ratio <0.767 or >1.3) (Sup-
plementary Materials, Table S2). With Proteome Discover 1.4
software, the distribution of significantly changed proteins
detected in ascites samples was illustrated in a volcano plot
(t-test p < 0.05, ratio >1.3 or < 0.767, Figure 1(a)) and the
expression levels of all proteins in each sample were visualized
in a hierarchical clustering heatmap (ratio >1.3 or < 0.767,
Figure 1(b)). The green dots in Figure 1(a) and cluster A in
Figure 1(b) indicate the downregulated proteins in HCC vs.
HD while the red dots and cluster B indicate the upregu-
lated proteins. With more strict criteria for significance (t-
test p < 0.05, ratio >1.5 or < 0.667), a total of 27 proteins
were identified as the differentially distributed proteins in
HCC vs. HD comparison, including 19 upregulated proteins
and 8 downregulated proteins. The top ranked differentially
distributed proteins are listed in Table 1. To further validate
the effectiveness of the iTRAQ analysis, ELISA assay was
employed to determine the concentrations of carbonic anhy-
drase I (CA1) in the ascitic fluid samples. The results indicated
that the level of CA1 in HCC was significantly lower than
that in HD group (Figure 1(c)), which was consistent with the
results from iTRAQ analysis (Table 1).

3.2. KOG and GO Classifications. In the following studies,
all 110 proteins identified in the above data were classi-
fied using KOG and GO analyses to predict their possible
roles in malignant ascites. The KOG database is a phylo-
genetic classification of the gene products from completely
sequenced genomes [20]. Annotated proteins in KOG are
assumed to originate from ancestor proteins, which reflect
the system evolution relationships of the individual proteins.
In our KOG functional classification, all the differentially
distributed proteins from HCC vs. HD were annotated into
16 KOG categories (Figure 2(a)). The top ranked functional
clusters included 3 largest categories in cellular processes
and signaling group (signal transduction mechanisms, defense
mechanisms and posttranslational modification, protein
turnover, and chaperones) followed by 2 smaller categories
in metabolism group (amino acid transport and metabolism,
inorganic ion transport and metabolism).

GO analysis is widely used to describe molecular function
of protein sets [21]. In our functional GO analysis, all of the
differentially distributed proteins were mapped to terms in the
GO database. The results showed the proteins belonged to
47 categories grouped into 3 major clusters including bio-
logical process, cellular components, and molecular function
(Figure 2(b)). The most commonly enriched GO terms of
HCC specific proteins were extracellular region and extracel-
lar region part.

The KOG and GO analyses indicated that protein signal-
ing transduction and extracellular region are major affected
function involved in HCC malignant ascites. These data
indicated that most of the differentially distributed proteins
between HCC ascites and HD ascites are likely membrane
proteins that are able to bind with specific ligand and activate
the signaling transduction.
**Figure 1:** Differentially distributed proteins identified by iTRAQ quantitative analysis. (a) Volcano plots showing differentially distributed proteins comparing HCC and HD. Each point represents the difference of fold-change (X axis, Log_{10}[fold change]) plotted against the level of statistical significance (Y axis, Log_{10}[p value]). Proteins represented by red dots indicate upregulation and green dots indicate downregulated proteins (p<0.05, ratio >1.3 or < 0.767). (b) Hierarchical clustering of the differentially distributed proteins identified from each samples (ratio >1.3 or < 0.767). The color scale indicates the expression level of each protein across the two groups. (c) ELISA assay for carbonic anhydrase I (CA1) in the ascitic fluid samples.

3.3. **KEGG Pathway Identification.** Kyoto Encyclopedia of Genes and Genomes (KEGG) is a database resource designed for characterization the high-level biological functions based on proteomic data. KEGG pathways were constructed to better understand the biological pathways and acquire the molecular mechanisms involved in HCC malignant ascites development. A summary of overall KEGG pathways (Table 2), KEGG pathways associated with upregulated (Figure 3(b), left) proteins and downregulated (Figure 3(b), right) differentially distributed proteins, was provided. KEGG pathway analysis of all the differentially distributed proteins found four enriched pathways with enrichment factor (the differentially distributed protein number/total protein number) higher than 0.75, including glycolysis/ gluconeogenesis pathway, carbon metabolism, and biosynthesis of amino acid pathway (all p<0.05). All of the 4 pathways are activated in HCC malignant ascites and associated with the upregulated proteins in HCC malignant ascites. The most inactivated pathway represented by downregulated protein is complement and coagulation cascades (p<0.10).

3.4. **Signal Pathway Analysis.** The glycolysis/gluconeogenesis pathway was found significantly activated in HCC ascites, including significantly upregulated proteins like Enolase-1 and phosphoglycerate kinase 1. These proteins were involved...
**Figure 2**: COG functional categories and GO classification. (a) Functional assignments of the differentially distributed proteins to the KOG (Eukaryotic Orthologous Groups) categories. The main KOG categories are illustrated with different colors and the frequency of each category is shown. (b) Overall GO (Gene Ontology) analysis of all differentially distributed proteins (HCC vs. HD) in accordance with the biological processes (left), cellular component (middle), and molecular function (right). Protein numbers in each category are shown.
Table 1: Top upregulated and downregulated proteins identified by iTRAQ (HCC vs. HD, p<0.05).

| Accession | Description/Gene name (Protein name) | Ratio (HCC/HD) | Score |
|-----------|--------------------------------------|----------------|-------|
| B4E1B2    | cDNA FLJ53691                         | 1.60           | 4358  |
| Q6MZU6    | DKFZp686C15213                        | 2.21           | 3984  |
| A2MYE1    | VACWR153(A30 protein)                 | 1.55           | 493   |
| A2JIN3    | Rheumatoid factor RF-IP20             | 2.72           | 332   |
| A8K9J7    | H2B(Histone H2B)                      | 1.97           | 121   |
| H6VRF8    | KRT1(Keratin-1)                       | 1.88           | 110   |
| B0AZL7    | cDNAFLJ79457(highly similar to Insulin-like growth factor-binding protein) | 1.66 | 62 |
| A0N8J1    | V(k)3 sequence of NG9 gene from fetal liver DNA | 1.52 | 57 |
| P35908    | KRT2(Keratin-2)                       | 1.53           | 51    |
| Q04756    | HGFA(Hepatocyte growth factor activator) | 1.59 | 45 |

| Accession | Description/Gene name (Protein name) | Ratio (HCC/HD) | Score |
|-----------|--------------------------------------|----------------|-------|
| Q6PIQ7    | IGL(Immunoglobulin lambda)           | 0.61           | 3129  |
| Q6DHW4    | Uncharacterized protein               | 0.60           | 526   |
| A0A0J9YXX1| Uncharacterized protein               | 0.43           | 146   |
| E5RIF9    | CA1(Carbonic anhydrase 1)            | 0.57           | 104   |
| Q5NV65    | V1-5(V1-5 protein)                   | 0.64           | 45    |

in metabolism of glycolysis, which is known for a potent driving force of tumor growth and therapy failure (Figure 4(a)). Another pathway coagulation and complement cascade was also focused here due to its central function in controlling fibrin clot formation. This pathway exhibits the highest number of assigned differentially distributed proteins identified in the above data. Many coagulation products, such as fibrinogen, antithrombin, plasminogen, and vitronectin, were downregulated in HCC vs. HD (Figure 4(b)). These results suggested the HCC ascites specific proteins reflect a response to local tumor microenvironment and could serve as an important attribute of disease pathogenesis.

4. Discussion

To the best of our knowledge, here we reported for the first time the proteomic profiling of HCC ascites using iTRAQ-based proteomic profiling, which is of high prognostic importance and a valuable tool to identify biomarkers not only to distinguish the malignant HCC from benign ascites caused by other chronic liver diseases, but also to predict disease progression during ascites formation. More importantly, a few functional categories and key factors were found affected in HCC ascites, indicating the potential of these molecular signatures as biomarker for diagnosis and prognosis. In our study, 112 differentially distributed proteins (69 upregulated and 43 downregulated) were characterized from ascitic fluid samples of HCC patients, including keratin 1 (KRT1), keratin 2 (KRT2), carbonic anhydrase 1 (CA1), and hepatocyte growth factor activator (HGFA).

With the increasing complexity of HCC, scientists have to explore new biomarkers to meet the demands from clinical diagnosis. Although the molecular mechanisms by which HCC develops remain unknown due to its heterogeneity, a multitude of proteomic, pathological, and molecular signatures of HCC have been uncovered and modeled to predictive or diagnostic biomarker. iTRAQ technique has been used to discover new protein biomarkers in almost every field of clinical diagnosis and translational research. Patients with chronic liver disease can present with acute decompensation due to various causes, including benign ascites and HCC [22]. Compared to serum or plasma based biomarker, proteomic analyses of body fluid could directly evaluate the local liquid microenvironment of cancer cells, which is a major reason why we chose to identify biomarkers for HCC. Our data also provided additional proof that the iTRAQ technique is capable of quantifying the protein levels change from ascitic fluid samples.

One of the gold standards for HCC diagnosis is alpha-fetoprotein (AFP), which is widely used in China and other Asian countries [23]. Recent studies have identified some other protein biomarkers which are able to be supplementary to AFP in the detection of HCC, such as Glypican-3 (GPC-3), Osteopontin (OPN), Golgi protein-73, squamous cell carcinoma antigen (SCCA), Annexin A2, and Thioredoxins [24, 25]. Most of the current protein biomarkers for HCC were focused on blood based specimen and showed low specificity for diagnosis or progression prediction. Similar to our study, a new protein biomarker S100A9 recently was identified to be upregulated in the tumor tissue interstitial fluids (p<0.05, ratio >1.3) [26]. ROC analysis showed this protein has sensitivity of 91% (higher than AFP) and specificity of 66% when used to distinguish HCC from liver cirrhosis (LC, HCC high risk population). The proteins
Figure 3: KEGG pathways in HCC ascites samples compared to IHD. (a) The most significantly enriched KEGG (Kyoto Encyclopedia of Genes and Genomes) pathways are illustrated with bubbles. The Y axis indicated the enrichment factor, which refers to the ratio of the differentially distributed protein number to the total protein number in a certain pathway. The size of bubble indicates mean number of proteins enriched in a given pathway. The color of bubble indicates Q value (adjusted p value). (b) Bar plots represent enriched KEGG pathways associated with upregulated (left) proteins and downregulated (right) differentially distributed proteins. The Y axis indicated average change fold of the implicated proteins.
Table 2: Top upregulated and downregulated KEGG pathways identified by iTRAQ (HCC vs. HD).

| Pathway ID | Pathway Name                                         | Gene Name            |
|------------|------------------------------------------------------|----------------------|
| ko00010    | Glycolysis / Gluconeogenesis (p<0.05)                | GAPDH, ENO1, PGK1    |
| ko01200    | Carbon metabolism (p<0.05)                           | GAPDH, ENO1, PGK1    |
| ko01230    | Biosynthesis of amino acids (p<0.05)                 | GAPDH, ENO1, PGK1    |
| ko00710    | Carbon fixation in photosynthetic organisms          | GAPDH, ENO1          |
| ko04066    | HIF-1 signaling pathway                              | GAPDH, ENO1          |
| ko00680    | Methane metabolism                                  | ENO1                 |
| ko03018    | RNA degradation                                      | ENO1                 |
| ko04015    | Rap1 signaling pathway                               | PFN1                 |
| ko04080    | Neuroactive ligand-receptor interaction              | CTSG                 |
| ko04142    | Lysosome                                             | CTSG                 |
| ko04145    | Phagosome                                            | MPO                  |
| ko04614    | Renin-angiotensin system                             | CTSG                 |
| ko04672    | Intestinal immune network for IgA production        | PIGR                 |
| ko04810    | Regulation of actin cytoskeleton                     | PFN1                 |
| ko05010    | Alzheimer’s disease                                  | GAPDH                |
| ko05131    | Shigellosis                                          | PFN1                 |
| ko05132    | Salmonella infection                                 | PFN1                 |
| ko05146    | Amoebiasis                                           | CTSG                 |
| ko05202    | Transcriptional misregulation in cancer              | MPO                  |
| ko05322    | Systemic lupus erythematosus                         | CTSG                 |

| Pathway ID | Pathway Name                                         | Gene Name            |
|------------|------------------------------------------------------|----------------------|
| ko04610    | Complement and coagulation cascades (p<0.10)         | CO4B, FIBA, FGB ANT3, PLMN, VTNCA2AP, IPSP, Q8IZZ5 |
| ko04611    | Platelet activation                                  | FGA                  |
| ko05150    | Staphylococcus aureus infection                      | C4B                  |
| ko04080    | Neuroactive ligand-receptor interaction              | PLG                  |
| ko04151    | PI3K-Akt signaling pathway                           | VTNC                 |
| ko04510    | Focal adhesion                                       | VTNC                 |
| ko04512    | ECM-receptor interaction                             | VTNC                 |
| ko05133    | Pertussis                                            | CO4B                 |
| ko05164    | Influenza A                                          | PLG                  |
| ko05205    | Proteoglycans in cancer                              | VTNC                 |
| ko05322    | Systemic lupus erythematosus                         | CO4B                 |

Identified in the present study have shown strong oncogenic ability with HCC or even biomarker potential in previous studies, suggesting these profiling data fit with some current hypotheses. For instance, keratin family proteins such as keratin 19 indicate high risk of tumor metastasis, invasion, and poor prognosis in HCC patients [27, 28]. Carbonic anhydrase I was found to be downregulated in HCC samples by iTRAQ-based quantitative proteomic analysis, which was confirmed by ELISA (Figure 1(c)). Carbonic anhydrases function by maintaining acid-base balance in blood and other tissues and participating in transporting carbon dioxide out of tissues. Carbonic anhydrase was also found to be correlated with tumor progression and predicted poor survival of HCC patients with high tumor stage. The role of carbonic anhydrases in ascites formation progression still remains poorly understood. Characterization of the implication of carbonic anhydrases in HCC ascites might reveal underlying mechanisms through which malignant ascites were generated, and carbonic anhydrases could potentially serve as ascites based biomarkers for HCC.

Over the past decade, the importance of the tumor microenvironment in HCC progression has been recognized but has not been well defined yet. It has been demonstrated that the malignant cells and the molecular signatures of ascites are changed continuously during the course of HCC [29]. It is not surprising that KOG and GO analyses on the presented data demonstrated that several key cellular functions were affected in HCC ascites, including signal
transduction mechanisms, defense mechanisms and post-translational modification, protein turnover, and chaperones. In addition to that, key metabolic processes including amino acid transportation and inorganic ion transportation were enriched in HCC group compared to HD control group. These findings were suggestive of the underlying mechanisms that regulate tumor cell growth and cellular metabolism in malignant ascetics and may help elucidate the molecular basis of HCC progression.

The signaling pathway signatures we reported here will shed light on the biomarker discovery in ascites and other inflammatory fluid. In particular two pathways were highlighted in the current study. Enzymes such as Enolase-1 and phosphoglycerate kinase in glycolysis/gluconeogenesis pathway were found significantly activated in HCC ascites. Our data is consistent with previous reports that Enolase-1 is expressed remarkably differently between the HCC tissue samples and precancerous lesions [30]. Moreover, serum antibodies against Enolase-1 are potential biomarkers for predicting Enolase-1 in HCC prior to surgical resection [31]. These biomarkers should be further investigated as potential therapeutic targets. These results indicated that HCC ascites specific proteins such as Enolase-1 responded and altered proteostasis in the unique cellular environment of HCC malignant ascetics and may predict HCC progression or be used as a potential therapeutic target for HCC.

Our study also suggested that coagulation and complement pathway is implicated in HCC malignant ascites formation, and the coagulation products (such as fibrinogen and plasminogen) might indicate status of HCC progress. Serum fibrinogen level is positively correlated with advanced tumor stage and poor survival in gastric cancer patients, suggesting fibrinogen protein as a biomarker for predicting tumor progression and survival of the patients [32]. Agreeing with our data, increased tendency to hemorrhage was observed in cancer patients [33]. A previous study reported a scoring system using combination of fibrinogen concentration and neutrophil-lymphocyte ratio to predict tumor progression in gastric cancer [34]. Circulating fibrinogen was found to be a prognostic and predictive biomarker in malignant pleural mesothelioma [35]. The similar findings were also reported in lung cancer, cervical cancer, prostate cancer, and breast cancer [36–38]. A very recent report found high fibrinogen level in plasma is significantly associated with poorer overall
survival of HCC patients with or without radical therapies [39]. Consistent with that, our study showed fibrinogen level was decreased in HCC ascites compared with benign ascites, suggesting its potential to predict prognosis of the patients with HCC.

Taken together, the analysis of malignant ascites has identified numerous potential biomarkers that could provide more information underlying HCC metastasis and progression. Some potential biomarker, such as Enolase-1 in glycolysis/gluconeogenesis pathway and fibrinogen in coagulation and complement cascades pathway, as discussed above, should be further determined in additional samples to validate the results presented in this study. Further investigation in a larger study population with a rigorous selection of the candidate proteins is needed in order to validate the clinical values of the biomarkers. Efforts should be directed towards prospective clinical trials in evaluating the prognostic significance of these candidate markers.

5. Conclusion

Utilizing iTRAQ-based proteomics analysis, the profiles of malignant ascites from HCC patients were characterized by identifying the differentially distributed proteins compared with benign ascites. Glycolysis/gluconeogenesis and complement/coagulation cascades are remarkably affected in HCC malignant ascites, which strongly suggests the protein molecules involved in these pathways such as Enolase-1 and fibrinogen are potential ascitic fluid based biomarkers for not only establishing diagnosis but also predicting clinical outcomes for HCC.

Data Availability

The data in an Excel file used to support the findings of this study are included within the Supplementary File S1.

Disclosure

The funders were not involved in the manuscript writing, editing, approval, or decision to publish.

Conflicts of Interest

The authors declare no competing financial interests.

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Supplementary Materials

Supplementary Table S1: characteristics of the 19 enrolled patients are shown.

Supplementary Figure S1: iTRAQ labeling strategy and study protocol are illustrated.

Supplementary Figures S2: histograms above indicate the iTRAQ-based quantification ratio (HCC vs. HD) distribution. X axis: Log2 (fold-change); Y axis: frequency (sample number).

Supplementary File S1: data of quantitative proteomic analysis for a total of 627 proteins detected in this study is shown.

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