Shotgun Proteomics Analysis of Differentially Expressed Urinary Proteins Involved in the Hepatocellular Carcinoma

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Abstract

Numerous investigations underlying the hepatocellular carcinoma (HCC) diagnosis as well as detection at an early stage based on hepatitis B virus surface antigen (HbsAg) concentration in serum and aflatoxin metabolites in urine have been commonly reported in the literature. To date, these biomarkers, even though specific and accurate, are not universal for the detection of HCC elicited by all causative factors. In addition, potential biomarkers may be present at low concentrations in contrast to the presence of abundant interfering proteins with a wide dynamic range. The aim of this study is to establish an operational analysis platform of effective and noninvasive diagnostic tool with a high sensitivity to explore protein expression profiles by shotgun proteomics using nano-liquid chromatography coupled tandem mass spectrometry (nanoLC-MS/MS) and stable isotope dimethyl labeling. Differentially expressed urinary proteins were identified and compared by the mass spectral patterns of their peptide fragments generated from protease digestion. In our results, the quantitative proteomic analysis of the differentially expressed proteins in urine identified at least 21 protein biomarker candidates with high confidence levels. We have further identified 14 proteins with up-regulation (stable isotope D/H ratio ≥ 1.5) and 7 with down-regulation (D/H ratio ≤ 0.6). The systematic decrease or increase of these identified marker proteins may potentially reflect the morphological aberrations and diseased stages of liver throughout progressive developments of HCC. The results would place a firm foundation for future work regarding validation and clinical translation of some identified biomarkers into targeted diagnosis and therapy for various classes of HCC.

Keywords: Quantitative proteomics; Hepatocellular carcinoma (HCC); Shotgun proteomics analysis; Nano-liquid chromatography coupled tandem mass spectrometry (nanoLC-MS/MS); Stable isotope dimethyl labeling

Introduction

Hepatocellular carcinoma (HCC), being ranked as the seventh most common malignant tumor in women and the fifth in men worldwide [1], is the first leading cause of cancer death in epidemiological studies of Taiwan in recent decades [2]. It has also been noted that HCC was prevalent in middle and western Africa, and eastern and south-eastern Asia; furthermore, over 80% of HCC incidences occur in developing countries [1]. Many etiological factors accountable for a wide spectrum of clinical manifestations were found to be associated generally with chronic infection with hepatitis B or C virus (HBV, HCV) and cirrhosis [3]. Moreover, several environmental factors including alcoholism, tobacco smoking and dietary exposure to aflatoxins could also account partly for a high incidence of HCC [4,5]. Many patients detected with HCC were found to have cirrhosis when they were initially diagnosed as sufferers of chronic liver disease lacking long-term clinical care and appropriate treatment. This abnormal cirrhosis condition results in an increase in the replacement of normal tissue with fibrous tissue leading to the loss of functional liver cells and the development of HCC [6].

The mortality rate for HCC exceeds 30 cases per 10,000 population and most cases are resistant to traditional chemotherapy and radiotherapy [7]. A wide variety of chemotherapeutic agents currently in use include fluorouracil, doxorubicin, mitoxantrone, cisplatin, mitomycin C, epirubicin, interferon-alpha and tegafur. However, no curative regimen has been found to date. The drug response and prolongation of survival are usually minimal (a few months or less), and there is a significant morbidity associated with poor treatment [8]. Curative surgery of HCC is feasible for only about 30% of patients. Transarterial embolization or chemoembolization (TAE/TACE) has been demonstrated to provide some survival benefits if tumors are confined to the localized area of liver and no evidence of portal vein thrombosis is found [9]. Therefore, in general diagnosis or detection at an early stage of HCC is considered to be essential to allow favorable and positive clinical treatments for increasing the life expectancy of HCC patients. Some screening tools such as the measurement of serum alpha-fetoprotein (AFP) or an abdominal ultrasonography at regular intervals targeting high risk populations were also applied to the detection of HCC at an early stage. Unfortunately, poor sensitivity and specificity of AFP and the need of an operator’s expertise required on the ultrasonographic evaluation limited their prognostic use.

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Combined use of AFP testing and ultrasonography was also reported to increase false-positive rates [10]. Other tests, including Lens culinaris agglutinin-reactive AFP and des-gamma carboxyprothrombin (DCP), are currently being evaluated and validated [11]. Owing to the lack of accurate and specific biomarkers for the assessment of HCC incidence at an early stage, HCC is thus considered to be difficult for detection and usually diagnosed as an incurable disease when diagnosed at a late diseased stage. Therefore it is very urgent and imperative to discover biomarkers with prognosis potential coupled with the development of curative therapy in the effective management of HCC.

Although greater emphasis in biological research is being directed toward a comprehensive global analysis of protein expression profiles for biomarker discovery, reliable and high-throughput proteome-wide comparative analyses for some diseases has not existed until the advent of current proteomics instrumentation. The qualitative and quantitative studies of proteins by means of fast-evolving and state-of-the-art proteomics methodologies have provided a firm basis for understanding the complex proteome profiles of total protein mixtures from various sample sources such as tissues, cells, plasma, and urine [12,13]. A major phase of various proteomics strategies lies in the determination of protein identity (Protein ID) of interest using analytical “fingerprints” or peptide mass fingerprinting (PMF) generated by digestion of proteins with cleavage-specific enzymes such as trypsin or some other well-characterized proteases, from which tandem mass (MS/MS) spectra of peptide fragments can then be used for comparison and confirmation of protein ID in available sequence databases. The strategy based on “bottom-up” proteomic approach by means of the direct analysis of peptides generated from protein digestion by high-resolution liquid chromatographies coupled with tandem MS/MS spectrometry (nanoLC-MS/MS) has facilitated the so-called “shotgun proteomics” for the identification of protein mixtures from any tissues of interest. Basically shotgun proteomics detects PMF profiles throughout the whole cellular proteome based on a random statistical sampling method similar to that of shotgun genomics in the decoding of human genome sequences. Various MS/MS spectra can be algorithmically compared with predicted peptide spectra from sequence databases to identify the respective proteins. This shotgun proteomics approach is capable of characterizing proteins directly from entire tissue or cell lysates [14-16].

In this study, we aim to establish a high-throughput operation platform of effective and noninvasive diagnostic tool for early detection of HCC biomarkers. To attain this goal, we have made an effort to characterize and compare the urinary proteins between diseased and control groups in order to identify potential biomarker candidates by means of gel-free shotgun proteomic analysis coupled with stable isotope dimethyl labeling [12,16,17] and nanoLC-MS/MS [17-19]. The proteomic analysis at the global cellular level reported herein will lay a firm foundation for future work regarding validation and clinical translation of some identified biomarkers into targeted diagnosis and therapy for HCC.

Materials and Methods

Trichloroacetic acid (TCA), trifluoroacetic acid (TFA), dithiothreitol (DTT), iodoacetamide (IAM), ethylenediaminetetraacetic acid (EDTA), sodium deoxycholate, sodium fluoride (NaF), formic acid-H$_2$O, formaldehyde-H$_2$O, and ammonium bicarbonate (NH$_4$HCO$_3$). Triton X-100 were purchased from Sigma Aldrich (St. Louis, MO). Acetonitrile (ACN) and sodium phosphate were obtained from Merck (Darmstadt, Germany). Formic acid (FA), sodium acetate, sodium cyanoborohydride and sodium chloride (NaCl) were purchased from Riedel-de Haven (Seelze, Germany). Protease inhibitors (Complete™ Mini) were purchased from Roche (Mannheim, Germany). Sodium dodecyl sulfate (SDS) and urea were purchased from Amresco (Solon, OH). Modified sequencing-grade trypsin for in-gel digestion was purchased from Promega (Madison, WI). Quantitative reagent for protein contents was purchased from Bio-Rad (Hercules, CA). Water was deionized to 18 MΩ by a Milli-Q system (Millipore, Bedford, MA).

Sample collection

All the procedures used in this study were approved by the ethical committee of clinical research at Kaohsiung Medical University Hospital. We collected urine from patients who were diagnosed as cases of HCC incidence and never underwent cholecystectomy as the disease group. We also got agreement from patients diagnosed with non-HCC incidence but underwent cholecystectomy for urine collection as the normal control. 50 mL urine for each individual were harvested, concentrated by centrifugation and assayed for determination of total protein concentration using Coomassie protein assay reagent, and subsequently were stored at -80°C until being analyzed.

Dimethyl labeling and peptide preparation

Volumes of urine containing 100 μg of total proteins were adjusted to 60 μL and treated with 0.7 μL of 1 DTT and 9.3 μL of 7.5% SDS at 95°C for 5 min before reduction. After the reaction, lysates were further treated with 8 μL of 50 mM IAM at room temperature for alkylation in the dark for 30 min; subsequently proteins were precipitated by adding 52 μL of 50% TCA and incubated on ice for 15 min. After removing the supernatant by centrifugation at 13,000 x g for 5 min, the collected proteins were washed with 150 μL of 10% TCA, vortexed and centrifuged at 13,000 x g for 10 min. The precipitated proteins were washed again with 250 μL distilled H$_2$O, vortexed and centrifuged thrice under the same condition. The resultant pellets were resuspended with 50 mM NH$_4$HCO$_3$ (pH 8.5), then digested with 4 μL of trypsin for 8 h at 37°C and further dried in a vacuum centrifuge to remove NH$_4$HCO$_3$. The lyophilized peptides from HCC and normal urine re-dissolved in 180 μL of 100 mM sodium acetate at pH 5.5 were treated with 20 μL of 4% formic acid-H$_2$O and 20 μL 4% formaldehyde-D$_2$, respectively [17-19] and mixed thoroughly. The mixtures were vortexed for 5 min, immediately followed by the addition of 10 μL of 0.6 M sodium cyanoborohydride and vortexed for 1 h at room temperature. The resultant liquids were acidified by 10% TFA/ H$_2$O to pH 2.0–3.0 and applied onto the in-house reverse-phase C18 column pre-equilibrated with 200 μL of 0.1% TFA/H$_2$O (pH 2.0–3.0) for desalting. The column was also washed with 200 μL of 0.1% TFA/H$_2$O (pH 3.0) and then eluted with a stepwise ACN gradient from 50% to 100% in 0.1% TFA at room temperature.

Hydrophilic interaction chromatography (HILIC) for peptide separation

HILIC was performed on an L-7100 pump system with quaternary gradient capability (Hitachi, Tokyo, Japan) using a TSK gel Amide-80 HILIC column (2.0×150 mm, 5 μm; Tosoh Biosciences, Tokyo, Japan) [20-22] with a flow rate of 200 μL/min. Two buffers were used for gradient elution: solvent (A), 0.1% TFA in water, and solvent (B), 0.1% TFA in 100% ACN. The eluted fractions after being desalted from the in-house reverse-phase C18 column were each dissolved in 25 μL of solution containing 85% ACN and 0.1% TFA and then injected into the
Protein factors characterized by proteomic analysis were analyzed for their association with mapping related to canonical pathways deposited in the IPA library.

Results and Discussion

Protein expression levels analyzed by nanoLC-MS/MS

Quantitative proteome analysis by shotgun approach coupled with stable isotope dimethyl labeling has been used in identifying candidate biomarkers or target factors in different types of samples on account of the fact that this approach can detect differentially released proteins at relatively low abundance [28-31]. In this study, we conducted a comparative proteomics investigation of urine between HCC patients and control group by a bottom-up shotgun proteomic approach. A schematic representation of sample processing, separation and the subsequent workflow concerning trypsin digestion, dimethyl labeling and shotgun analysis is shown in Figure 1. Initially, 100 μg each of total urine proteins from HCC patients and control group was subjected to trypsin digestion and dimethyl labeling. Respective tryptic peptide samples were mixed in a 1:1 (w/w) ratio and then enriched by the reverse-phase C18 column. Owing to the fact that the enriched peptide population was too complex to be fully detected and characterized by a single LC-MS/MS run, the enriched peptides were fractionated by HILIC based on polarity difference, and then harvested into 10 fractions. Each fraction was analyzed by LC-LTQ-Orbitrap and the parameter used in searching for peptide identification was adjusted to allow for no missed cleavage. Most of the peptides were separated from a single or two adjacent HILIC fractions, and peptides identified by the Mascot search program (http://www.matrixscience.com) [23,24] were accepted if their individual ion score was higher than 20, which had been a cutoff point used for the lower-quality MS/MS spectra [32-34] Supplementary information.

Figure 1: Experimental scheme of the procedures used for the screening of differentially expressed proteins. After enzymatic digestion, peptides were differentially stable isotope dimethyl labeled and combined prior to desalting and fractionation. The quantitative shotgun analysis of proteome changes from clinical urine samples of normal and HCC patients was carried out by using HILIC-C18 peptide separation and nanoLC-MS/MS coupled with stable isotope dimethyl labeling.

20 μL sample loop. The gradient was processed as follows: 98% (B) for 5 min, 98-85% (B) for 5 min, 85-0% (B) for 40 min, 0% (B) for 5 min, 0-98% (B) for 2 min and 98% (B) for 3 min. A total of 10 fractions were collected (1.2 mL for each fraction) and dried in a vacuum centrifuge.

NanoLC-MS/MS analysis

The lyophilized powders were reconstituted in 10 μL of 0.1% FA in H₂O and analyzed by LTQ Orbitrap XL (Thermo Fisher Scientific, San Jose, CA). Reverse-phase nano LC separation was performed on an Agilent 12000 series nanoflow system (Agilent Technologies, Santa Clara, CA). A total of 10 μL sample from collected fractions was loaded onto an Agilent Zorbax XDB C18 precolumn (0.35 mm, 5 μm), followed by separation using in-house C18 column (i.d. 75 μm×15 cm, 3 μm). The mobile phases used were (A) 0.1% FA in water and (B) 0.1% FA in 100% ACN. A linear gradient from 5% to 95% of (B) over a 70-min period at a flow rate of 300 nL/min was applied. The peptides were analyzed in the positive ion mode by applying a voltage of 1.8 kV to the injection needle. The MS was operated in a data-dependent mode, in which one full scan with m/z 400-1600 in the Orbitrap using a scan rate of 30 ms/scan. The fragmentation was performed using the CID mode with collision energy of 35 V. A repeat duration of 30 s was applied to exclude the same m/z ions from the reselection for fragmentation. The Xcalibur software (version 2.0.7, Thermo Fisher Scientific, San Jose, CA) was used for the management of instrument control, data acquisition, and data processing.

Protein database search and characterization

Peptides were identified by peak lists converted from the nanoLC-MS/MS spectra by bioinformatics searching against Homo sapiens taxonomy in the Swiss-Prot databases for exact matches using the Mascot search program (http://www.matrixscience.com) [23,24]. Parameters were set as follows: a mass tolerance of 10 ppm for precursor ions and 0.8 Da for fragment ions; no missed cleavage site allowed for no missed cleavage. Most of the peptides were separated from a single or two adjacent HILIC fractions, and peptides identified by the Mascot search program (http://www.matrixscience.com) [23,24] were accepted if their individual ion score was higher than 20 (p<0.05).

Subsequently, the analysis of peptide quantification ratio (D/H) for normal (hydrogen labeling) and HCC (deuterium labeling) from urine was carried out by Mascot Distiller program (version 2.3, Matrix Science Ltd., London, U.K.) using the average area of the first 3 isotopic peaks across the elution profile. The Mascot search data as well as quantification resulting from each fraction were also merged by this program that combined the peptide ratios matching the same parameter used in searching for peptide identification was adjusted to allow for no missed cleavage. Most of the peptides were separated from a single or two adjacent HILIC fractions, and peptides identified by the Mascot search program (http://www.matrixscience.com) [23,24] were accepted if their individual ion score was higher than 20 (p<0.05).

Constitution of signaling pathways and network analysis of protein interaction

The software program (www.ingenuity.com) from Ingenuity Pathways Analysis (IPA, Ingenuity Systems, Redwood City, CA) was used for deriving the pathways and networks of protein interaction. Protein factors characterized by proteomic analysis were analyzed for their association with mapping related to canonical pathways deposited in the IPA library.
Quantification of identified proteins with differential expression

Once the differentially released proteins with confident identification based on dimethyl labeling, enzyme digestion and peptide mass fingerprinting (PMF) were completed, the peptide quantification ratio (D/H) was obtained by Mascot Distiller program using the average area of the first 3 isotopic peaks across each elution profile [17,18,35]. Data from the publicly available Mascot searching engine as well as quantification results from each fraction were also merged by Mascot Distiller program into one file that combined peptides with more than one peptide matching the same sequence, which were harvested from different fractions or at different retention time and charge state. Herein, the 14 up-regulated (D/H ratio ≥ 1.5) and 7 down-regulated (D/H ratio ≤ 0.6) proteins displayed in at least four of seven comparative urine samples between HCC patients and control group were identified and listed in Table 1. The representative protein peaks including protein AMBP, prostaglandin-H₂, D-isomerase (PTGDS), serotransferrin (TF) and alpha-1-antitrypsin (SERPINA1) are shown in Figure 2-5. On the one hand, the isotopic pair (quantification ratio) of nano-LC and MS/MS CID spectra shown in Figure 2 demonstrate that D₄ and H₄-labeled peptides (VYAQGVPEDSIFTMADR) derived from protein AMBP, exhibiting m/z values of 1027.04 (+2) and 1025.02 (+2), respectively and D/H ratio of 10.32, were eluted at the same time (~19.1 min) in the HILIC fraction 5. D₄ and H₄-labeled peptides (TMLLQPAGSLGSYSYR) from PTGDS, showing m/z values of 896.47 (+2) and 894.45 (+2), respectively and D/H ratio of 5.36, were eluted at the same time (~18.27 min) in the HILIC fraction 5, as shown in Table 1. D-isomerase (PTGDS), serotransferrin (TF) and alpha-1-antitrypsin (SERPINA1) are shown in Figure 2-5. On the one hand, the isotopic pair (quantification ratio) of nano-LC and MS/MS CID spectra shown in Figure 2 demonstrate that D₄ and H₄-labeled peptides (VYAQGVPEDSIFTMADR) derived from protein AMBP, exhibiting m/z values of 1027.04 (+2) and 1025.02 (+2), respectively and D/H ratio of 10.32, were eluted at the same time (~19.1 min) in the HILIC fraction 5. D₄ and H₄-labeled peptides (TMLLQPAGSLGSYSYR) from PTGDS, showing m/z values of 896.47 (+2) and 894.45 (+2), respectively and D/H ratio of 5.36, were eluted at the same time (~18.27 min) in the HILIC fraction 5, as shown in Table 1.

### Table 1: Protein expression ratios of D/H shown here cover the range of seven measurements.

| Protein ID      | Gene symbol | D/H ratio | p-value | Number of sample pairs | pl/mass (kDa) | Number of peptides | Swiss prot accession number | Sequence coverage (%) |
|-----------------|-------------|-----------|---------|------------------------|---------------|-------------------|----------------------------|-----------------------|
| Protein AMBP    | AMBP        | 2.0 ~ 14.2 | 0.016*  | 6/7                    | 5.95 / 40.48  | 122               | P02780                     | 45                    |
| Prostaglandin-H₂ D-isomerase | PTGDS      | 1.6 ~ 11.8 | 0.0239* | 6/7                    | 7.66 / 21.56  | 23                | P41222                     | 42                    |
| Uromodulin      | UMOD       | 2.9 ~ 30.5 | 0.164   | 5/7                    | 5.05 / 72.96  | 90                | P07911                     | 44                    |
| Beta-2-glycoprotein 1 | APOH       | 2.4 ~ 35.8 | 0.101   | 5/7                    | 8.34 / 40.55  | 2                 | P02749                     | 14                    |
| Plasma serine protein inhibitor | SERPINA5 | 2.3 ~ 17.3 | 0.0417* | 5/7                    | 9.3 / 46.53   | 19                | P05154                     | 28                    |
| Lysosomal alpha-glucosidase | GAA        | 2.6 ~ 8.5  | 0.013*  | 5/7                    | 5.62 / 106.59 | 5                 | P10253                     | 17                    |
| Ig kappa chain V-II region SIE | KV302   | 1.6 ~ 18.1 | 0.216   | 5/7                    | 8.72 / 11.98  | 4                 | P01620                     | 39                    |
| Ig lambda-2 chain C | IGLC2      | 2.1 ~ 10.9  | 0.157   | 4/7                    | 6.92 / 11.69  | 17                | P0CG05                     | 69                    |
| Kininogen-1      | KNG1       | 2.7 ~ 28.4 | 0.108   | 4/7                    | 6.34 / 74.43  | 62                | P01042                     | 37                    |
| Basement membrane-specific heparan sulfate proteoglycan core protein | HSPG2     | 3.2 ~ 13.2 | 0.082   | 4/7                    | 6.06 / 481.43 | 35                | Q2VP1A                     | 10                    |
| Polymyric immunoglobulin receptor | PIGR     | 2.0 ~ 17.7 | 0.165   | 4/7                    | 5.58 / 85.78  | 5                 | P01833                     | 24                    |
| Ribonuclease     | RNASE1     | 4.5 ~ 30.3 | 0.099   | 4/7                    | 9.1 / 18.38   | 13                | P07998                     | 37                    |
| Ig kappa chain C region | IGKC     | 1.6 ~ 13.7 | 0.158   | 4/7                    | 5.58 / 12.00  | 62                | P01834                     | 80                    |
| Pro-epidermal growth factor | EGF       | 1.8 ~ 19.5  | 0.243   | 4/7                    | 5.53 / 139.41 | 2                 | P01133                     | 12                    |

### Table 2: Increased (D/H ratio ≥ 1.5) and decreased expression (D/H ratio ≤ 0.6) proteins were displayed in at least four of seven hepatocellular carcinoma (HCC) urine sample pairs when identified by nanoLC-MS/MS coupled with stable isotope dimethyl labeling.

**Protein expression ratios of D/H shown here cover the range of seven measurements.**

*Statistically significant data by student t-test (p<0.05)

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Figure 2: The MS/MS CID spectra and ratios of isotopic pair (quantification ratio) of the representative peptide VVAQGVIPEDSiFTMDR (A) and peptide quantification ratio (D/H) of HCC (deuterium labeling) and normal (hydrogen labeling) (B) for protein AMBP.

Figure 3: The MS/MS CID spectra and ratios of isotopic pair (quantification ratio) of the representative peptide TMLLPAGSGLGSYSVR (A) and peptide quantification ratio (D/H) of HCC (deuterium labeling) and normal (hydrogen labeling) (B) for prostaglandin-H2 D-isomerase (PTGDS).

Figure 4: The MS/MS CID spectra and ratios of isotopic pair (quantification ratio) of the representative peptide DGADVAVFK (A) and peptide quantification ratio (D/H) of HCC (deuterium labeling) and normal (hydrogen labeling) (B) for serotransferrin (TF).

Figure 5: The MS/MS CID spectra and ratios of isotopic pair (quantification ratio) of the representative peptide LSITGTYDLK (A) and peptide quantification ratio (D/H) of HCC (deuterium labeling) and normal (hydrogen labeling) (B) for alpha-1-antitrypsin (SERPINA1).
involved in the process of immune system (12%) showed reduced release, as shown in Figure 6D. Therefore, our current observation demonstrates that decreased and increased expression of these proteins implied the likelihood of their involvement in the pathologic statuses of carcinogenesis and differentiation of HCC cells.

**Construction of signaling pathways and network analysis of protein interaction**

Using a panel of these identified proteins, we further cluster them into a possible connection network based on the biochemical categorization to put forward a simulation scheme for the prospective signaling pathways governing the maintenance and progression of carcinogenic status in the liver tissue. In Figure 7, these identified proteins (shown in red color) mapped to canonical pathways derived by the Ingenuity Pathways Analysis (IPA, Ingenuity Systems) databank were displayed with different shapes to signify the disparate and diverse functions. Proteins reported in the literature and canonical pathway database based on their functional annotation were shown in white color and subjected to the association analysis and simulation of possible molecular interaction with our identified proteins. All the gray arrows designate the biological interrelationships between molecules. All arrows in the figure were supported by at least one reference from the literature, textbooks, or canonical information stored in the Ingenuity Knowledge Base. The increased release proteins including pro-epidermal growth factor (EGF), kininogen-1 (KNG1), beta-2-glycoprotein 1 (APOH) and polymeric immunoglobulin receptor (PIGR), to some extent, are involved in inflammatory responses; simultaneously, basement membrane-specific heparan sulfate proteoglycan core protein (HSPG2) and ribonuclease (RNASE1) were categorized to be involved in the proliferation of cancer cells. Namely, HCC was not characterized with a singular and clear-cut enzymatic or cytoskeleton alteration but with a series of complex and diverse functional changes.

Several proteins identified by our shotgun approach were not recruited to the canonical pathways mapped in the database owing to the fact that these proteins were not linked to functional interaction; however, the importance of these unmapped proteins with up-regulated release cannot be overlooked. The availability and suitability of these identified proteins employed as candidate biomarkers will be validated by subjecting them to next phases of verification and validation using ion scanning of peptides measured and quantified in multiple reaction monitoring (MRM) mode of nanoLC-MS/MS analysis. In addition, a much larger number of matched sample pairs should be essential for being able to discriminate the subtle and yet crucial differences of released proteins between HCC and corresponding normal counterparts. After completion of the initial phase of biomarker discovery based on limited sample pairs in this preliminary pilot study, we are currently embarking the second phase of biomarker verification based on the identified candidate marker proteins and an expanded scale of urine sample collection of HCC and some other patients of different diseases [37,38].

**Conclusion**

Hepatocellular carcinomas (HCCs) encompass different etiology and pathological manifestations coupled with heterogenic genomic alterations leading to high complexity and intractable therapy and treatment. Collectively, the severity of HCC involves a variety of protein factors which play some regulatory roles in metabolic coordination of physiological functions. The systematic decrease and increase of these proteins may be reflective of the dysfunction of liver cells, followed by morphological aberrations upon progressive developments of HCC. The comparative proteome data from urine samples may help not only offer a novel approach to further understand the mechanism(s) underlying the development and the associated metabolic signaling pathways entailed in liver carcinogenesis, but also develop potential and valuable biomarker candidates useful for the non-invasive diagnosis and prognosis.

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