View metadata, citation and similar papers at core.ac.uk

brought to you by CORE

provided by Elsevier - Publisher Connector

Kaohsiung Journal of Medical Sciences (2016) 32, 535—544

Available online at www.sciencedirect.com

ScienceDirect

journal homepage: http://www.kjms-online.com

REVIEW ARTICLE

Proteomic analysis and translational perspective of hepatocellular carcinoma: Identification of diagnostic protein biomarkers by an onco-proteogenomics approach

Shyh-Horng Chiou [a,b,c], King-Teh Lee [d,*]

[a] *Quantitative Proteomics Center and Graduate Institute of Medicine, Kaohsiung Medical University, Kaohsiung, Taiwan*
[b] *Institute of Biological Chemistry, Academia Sinica, Taipei, Taiwan*
[c] *Center for Infectious Disease and Cancer Research, Kaohsiung Medical University, Kaohsiung, Taiwan*
[d] *Division of Hepatobiliary Surgery, Department of Surgery, Kaohsiung Medical University Hospital, Kaohsiung Medical University, Kaohsiung, Taiwan*

Received 17 August 2016; accepted 8 September 2016
Available online 4 November 2016

KEYWORDS
Hepatocellular carcinoma;
Onco-proteogenomics;
Proteogenomics;
Urine proteomics

Abstract Hepatocellular carcinoma (HCC) has been ranked as the third leading cause of cancer-related mortality worldwide. Typically, patients are already in advanced stages of liver cirrhosis at the time of HCC diagnosis. Because HCC is often detected at a late stage and is highly aggressive, noninvasive biomarkers are urgently needed for early diagnosis. Recent advances in gene-expression profiling technologies have enabled molecular classification of HCC into defined subclasses that provide a firm basis for further study of potential mechanisms and biomarkers underlying the development of HCC. This study applied an integrated onco-proteogenomics approach to identify and characterize HCC biomarkers. Specifically, this study integrated proteomic, genomic, and transcriptomic methods to obtain protein expression profiles of urine and tissue samples from HCC patients and from normal controls. Two mediators of inflammation were positively identified: S100A9 and granulin protein markers, which belong to the cytoplasmic alarmin family of the host innate immune system. These HCC-associated

Conflict of interest: All authors declare no conflicts of interest.

* Corresponding author. Division of Hepatobiliary Surgery, Department of Surgery, Kaohsiung Medical University Hospital, Kaohsiung Medical University, No. 100, Shiquan 1st Rd., Sanmin District, Kaohsiung City 807, Taiwan.
E-mail address: ktlee@kmu.edu.tw (K.-T. Lee).

http://dx.doi.org/10.1016/j.kjms.2016.09.002
1607-551X/Copyright © 2016, Kaohsiung Medical University. Published by Elsevier Taiwan LLC. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

cancer-specific biomarkers may have contributing roles not only in the dysregulated processes associated with various inflammatory and autoimmune conditions, but also in tumorigenesis and cancer metastasis.
Copyright © 2016, Kaohsiung Medical University. Published by Elsevier Taiwan LLC. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

Introduction

Proteomic technology enables large-scale profiling of global protein expression and systematic monitoring of proteins and protein variation in the diverse landscape of cellular translation. Therefore, it enables in-depth analyses of host and pathogen interactions. The emergence of databases of complete human and animal genomes and computer modeling now enable protein-level analysis of proteomes in samples from diverse biomedical sources by coupling various proteomic strategies with state-of-the-art mass spectrometry (MS) [1,2]. The diverse and intricate protein components identified in proteomes of biological tissues and cells can then be compared with *in silico* peptide maps derived from enzyme-digested proteins or genomic databases deposited in assorted databanks. Thus, instead of using conventional analyses of protein chemistry for global characterization of cellular proteins, researchers are now combining proteomic/genomic analyses with fast-evolving MS instrumentation. Methods of characterizing and sequencing genomic alterations in various biological specimens are also rapidly improving. The next logical step is identifying functional changes in the human proteome caused by genomic alterations or other environmental factors and then performing clinical studies to identify and validate protein biomarkers of these changes.

Integrating advanced genomic analysis with proteomic characterization not only has great potential use for discovering useful biomarkers, but it may also drive the development of new diagnostics methods and therapies. Increasing use of these new approaches thus sets the stage for development of new molecularly targeted cancer interventions [3].

Tumor heterogeneity is widely considered the main cause of ineffective and unsuccessful cancer treatment. Therefore, an improved understanding of the effects of complex proteomes in cancer cell microenvironments is urgently needed [4]. Novel applications of proteomic technology, particularly in translational medicine, have been reported in clinical studies of cancer [5]. For example, coupling proteomic approaches with bioinformatic mining of the human cancer genome has proven useful for elucidating molecular mechanisms related to tumorigenesis. Many other novel applications of proteogenomic and onco-proteomic methods have been reported in the recent literature [6—9].

Hepatocellular carcinoma (HCC) is the third and the first leading cause of cancer-related mortality worldwide and in Taiwan, respectively [10,11]. A major etiological factor in HCC is cirrhosis, which is usually caused by chronic infection with hepatitis B or C virus, nonalcoholic fatty liver disease, and alcohol abuse [12]. Many HCC patients are initially diagnosed with chronic liver diseases, resulting in replacement of normal tissue with fibrous tissue [13] and gradual progression to an incurable stage of HCC. The widely varying pathological manifestations and etiologies of HCC as well as its high potential for multiple genomic aberrations have resulted in an obstinate treatment for HCC. The biggest therapeutic advance in the past decade, which is the introduction of the multi-kinase inhibitor sorafenib, only extends life expectancy by 8—11 months [14]. Because of its limited therapeutic efficacy, improved molecularly targeted therapies, such as therapies for inhibiting cyclin-dependent kinase 9 [15] or MET [16], are urgently needed. Moreover, barriers to developing effective therapies for HCC highlight the need for new noninvasive biomarkers for early detection and diagnosis/prognosis in this refractory class of liver cancer.

Current cancer diagnostic methods still rely on tests for classic cancer biomarkers (e.g., tumor antigen markers carbohydrate antigen-125, carbohydrate antigen 19-9, and carcinoembryonic antigen combined with histopathological analysis of tissue biopsy samples. The specificity and sensitivity of these methods are inconsistent [17]. Currently, abdominal ultrasonography and measurement of serum α-fetoprotein are the two main tools for detecting HCC at an early stage [18]. However, the expertise needed to perform abdominal ultrasonography and the poor sensitivity and specificity of α-fetoprotein have limited their diagnostic and prognostic use. By contrast, recent developments in proteomic technologies now enable reliable high-throughput identification of protein mixtures in biological tissues, which provides a good basis for understanding the complex proteomic profiles of various biological samples, including tissues, cells, plasma, and urine [19,20]. Moreover, qualitative and quantitative studies of proteins by rapidly evolving state-of-the-art proteomic methodologies such as shotgun proteomics enable comprehensive global analyses of protein expression profiles in various cancer types, including HCC [21—23]. Although researchers have used onco-proteomics to identify biomarkers, its use for identifying tumor-specific peptides is limited due to the complexities of tumor genomes and transcriptomes such as point mutations, copy-number aberrations, unusual splicing variants, and gene fusion [24,25]. Extensive genomic studies performed in recent years indicate that novel genomic amplifications, mutations, and deletions occur frequently in HCC [26,27]. For example, a novel HCC-promoting gene, FGF19, is reportedly amplified in HCC cells harboring the 11q13.3 amplicon [28]. Sawey et al [28] further showed that 11q13.3 amplification could be a useful biomarker of the response to anti-FGF19 therapy. Advances in gene-

expression profiling technologies have also enabled the molecular classification of HCCs into defined subgroups based on genomic and proteomic analysis data, which provide a good basis for performing more informative clinical trials [29]. Because it links cancer proteomics to genomics and transcriptomics, onco-proteogenomics is considered an important new research tool and strategy. By integrating and applying advanced methods of detecting various tumor-specific changes in the proteome, onco-proteogenomics can provide, an in-depth understanding of tumor initiation, progression, and responses to treatment [24,25,30]. By incorporating MS-generated data with genomic information, onco-proteogenomics also enables a comprehensive understanding of how cellular networks and canonical signaling pathways are dysregulated in various cancer types. Several previous studies have applied multiple proteogenomic strategies to characterize cell lines and primary tumors in colorectal, gastric and other cancer [30—32].

The objective of this study was to identify effective and noninvasive diagnostic biomarkers for early detection of HCC. Potential biomarker candidates were identified by characterizing and comparing urinary proteins between diseased and control groups by gel-free shotgun proteomic analysis coupled with stable isotope dimethyl labeling and nanoscale liquid chromatography—tandem MS (nano-LC—MS/MS) as reported previously [33—38]. We also report the use of our recently developed strategy for identifying biomarkers by combining conventional cancer genomics with rapidly evolving proteogenomic methods [24,25], that is, onco-proteogenomics. Using the proposed strategy to detect tumor-specific changes in human cancer genomes and transcriptomes provides important insights and enables a comprehensive understanding of how oncogenic signaling pathways are dysregulated in various cancer types such as human HCC, which we report herein, and gastric cancer, which we will report in a future study.

Basic MS-based proteomic methodologies and platforms

Sample collection

All procedures used in this study were approved by the Clinical Research Ethics Committee at Kaohsiung Medical University Hospital, Kaohsiung, Taiwan, and written informed consent was obtained from all participants. Forty patients who had been diagnosed with HCC but had never received cholecystectomy were enrolled in the disease group. Another 40 patients who had no history of HCC diagnosis but had received cholecystectomy were enrolled in the normal control group. Urine samples (50 mL) were collected from each participant and concentrated by centrifugation. After protein assay using Coomassie Protein Assay Reagent, each sample was stored at −80°C until further analysis.

Gel-free shotgun MS-based proteomics

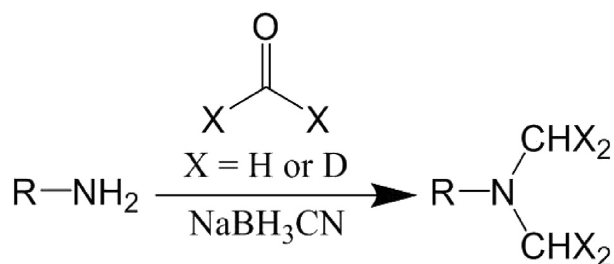
MS-based proteomic technology has progressed rapidly in the past decade. The main strategies for performing proteomic studies can be classified as bottom-up and top-down approaches. Bottom-up approaches characterize proteins by analyzing peptides released through proteolysis. By contrast, top-down approaches use direct electrospray ionization of proteins followed by gas-phase fragmentation for MS analysis [39—41].

A mixture of proteins, such as a whole cellular proteome, can be analyzed by a bottom-up approach known as shotgun proteomics. First, intact proteins are either chemically or enzymatically cleaved to peptides. The resulting peptides are typically separated by LC followed by MS/MS, which generates characteristic fragmentation patterns of peptides [42]. Peptides are identified by comparing the peptide MS/MS pattern with theoretical MS/MS patterns generated by *in silico* digestion of a protein database. Proteins are then identified by mapping the identified peptides back to proteins based on a user-defined protein database. Because peptides can be either uniquely assigned to a single protein or shared by more than one protein, the identified proteins require further scoring and grouping according to their peptides. Sophisticated computational tools have been developed to enable automatic analysis of vast amounts of data generated by shotgun proteomics.

For a complex protein mixture such as the entire cell lysates, multidimensional LC is the shotgun proteomic approach typically used to reduce sample complexity and to increase the dynamic range of protein identification [22,23]. Currently, multidimensional LC is routinely used for femtomolar detection and measurement of thousands of proteins, within several hours. In recent years, gel-free shotgun proteomics combined with LC-MS/MS has been the preferred method of analyzing complex samples because it is highly automated and has high throughput and sensitivity. Recently developed MS methods can be used not only to identify proteins in a mixture, but also to determine relative levels of protein expression in one or more samples. That is, gel-free shotgun proteomics has paved the way to quantitative proteomics by circumventing conventional semi-quantitative gel-based proteomic methods based on image analysis of protein spots on two-dimensional gels. In this study, protein expression in urine samples from HCC patients and normal controls was profiled by shotgun proteomics using nanoLC—MS/MS and stable isotope dimethyl labeling (described below), followed by protein database searches for, and characterization and quantification of, detected biomarkers.

Stable isotope dimethyl labeling-based quantitative proteomics

Isotope dimethyl labeling is applicable for any protein sample and, compared to other quantitative methods based on stable isotope labeling, is simpler, more reliable, and more cost-effective [43]. This proteomic method was developed in 2003 by Hsu et al [44]. As shown in the following chemical equation, heavy (deuterium) and light (hydrogen) isotope formaldehyde were reacted with primary amines (the N terminus and the side chain of lysine residues) in a peptide mixture to form Schiff bases.



Reduction of cyanoborohydride then generated dimethylamine groups as shown below.

Isotope dimethyl labeling provides high reaction efficiency and selectivity without major byproducts [45]. Triplex or even more labeling tags have been used in multiplex isotope dimethyl labeling methods developed to facilitate time course studies and comparisons of multiple states (e.g., dose—response curves), or multiple disease stages [46]. The multiplex isotope labeling strategy improves protein quantification accuracy by analyzing multiple protein samples in one experiment, which minimizes variation in LC separation and MS detection procedures [47,48].

Figure 1. Potential urinary biomarkers for HCC were identified by an onco-proteogenomic approach. Urine samples from patients diagnosed with HCC and from normal controls ($n = 44$) were analyzed by quantitative proteomics. Identified candidates were further analyzed and selected using genomic/transcriptomic approaches. HCC = hepatocellular carcinoma.

Stable isotope dimethyl labeling-based quantitative proteomics for monitoring cancer progression in personalized medicine

Trends in protein expression dependent on disease progression can be identified by signals in the MS1 spectra of individual isotope tags, which represent the protein expression levels in corresponding disease stages. A protein is identified by its exact peptide mass in the MS1 scan (precursor mass) and by its characteristic fragmentation patterns in the MS2 scan. In this study, peptide and fragment masses were identified by peak lists converted from the nanoLC-MS/MS spectra. Specifically, the Mascot program (http://www.matrixscience.com) was used for searches/comparisons against the *Homo sapiens* taxonomy in the SwissProt databases for exact matches in a bioinformatics database [49,50]. Bioinformatic analyses of signaling pathways, protein—protein interaction networks, and clustering of time-dependent up/downregulation protein markers were then used to elucidate mechanisms underlying the progression of the studied disease.

Gene ontology and pathway analysis

The Database for Annotation, Visualization and Integrated Discovery (DAVID) Bioinformatics Resources [51] were used to perform gene ontology (GO) analyses to determine which functional processes were differentially represented in the protein list obtained from quantitative proteomic analyses of urine samples from HCC patients and normal controls. The up- and downregulated proteins from proteomic analysis were used for GO analysis.

Genomic and transcriptomic data analysis

All cancer genome datasets and bioinformatics tools used to visualize different parameters in analyses of genomic and transcriptomic data were accessed through the MSKCC cBio Core homepage (www.cbioportal.org) [52]. Co-occurring gene amplifications in HCC were identified by analyzing the Cancer Genome Atlas (TCGA; 193 tumor samples). Comparison of copy number aberrations and gene expression was also based on available TCGA datasets for HCC.

Preliminary results obtained by shotgun quantitative proteomics and onco-proteogenomic analyses

Many studies have investigated methods of diagnosis and early detection of HCC based on hepatitis B virus surface antigen concentrations in serum and in urinary aflatoxin metabolites. Several epidemiological studies have indicated that environmental factors, including alcohol use, tobacco use, and dietary exposure to aflatoxins, at least partly explain the high incidence of HCC [53]. Some biomarkers that have shown high specificity and accuracy for detecting HCC are not applicable for all environmental and genetic causal factors in HCC [54]. Additionally, potential biomarkers may be present at low concentrations whereas interfering proteins may be abundant in a wide dynamic range.

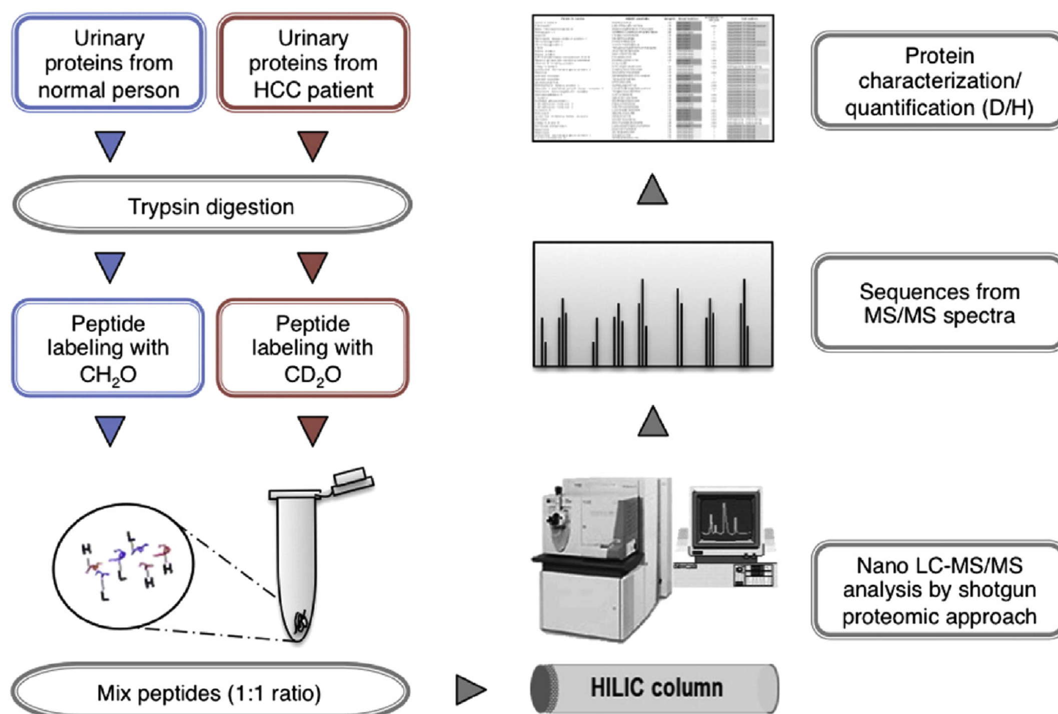


Figure 2. Flow-chart and experimental schemes of the procedures used for quantitative proteomics. Upon enzymatic digestion, peptides were differentially stable isotope dimethyl-labeled and combined prior to desalting and fractionation. The quantitative shotgun analysis of proteome changes in clinical samples of urine from hepatocellular carcinoma patients and normal controls was performed by hydrophilic interaction liquid chromatography (HILIC)-C18 peptide separation and nano-liquid chromatography—tandem mass spectrometry coupled with stable isotope dimethyl labeling.

Our recent pilot study showed some success in using an onco-proteogenomic approach to compare biomarkers from tissues and urine of HCC patients and normal controls [55]. The current study used an optimized shotgun-based quantitative proteomic strategy to analyze urinary proteins in each of 44 pairs of HCC patients and normal controls. Further cross-analyses of these proteomic results with genomic and transcriptomic data (Figure 1) revealed urinary HCC biomarkers with potential use individually or in combination with other biomarkers. As Figure 2 shows, two major protein biomarker candidates were identified with high confidence levels in quantitative proteomic analysis of differentially expressed proteins in urine samples from HCC patients.

Quantitative proteomic analysis of differentially expressed proteins in urine samples from HCC patients by a shotgun approach

Figure 2 shows that urinary proteins were characterized and compared between the disease and control groups ($n = 44$) by using gel-free shotgun proteomic analysis coupled with stable isotope dimethyl labeling [23,44] and nanoLC-MS/MS [46]. Differentially released proteins were identified by dimethyl labeling, enzyme digestion, and peptide mass fingerprinting followed by comparison of urinary-peptide quantification ratios (D/H) between HCC patients (deuterium labeling) and normal controls (hydrogen labeling). Approximately 1000 proteins were systematically mapped in the 44 pairs of urine samples. Figure 3 shows that expression levels of 91 identified proteins significantly differed ($p < 0.05$). Of these, 83 were upregulated (red shaded areas), and eight were downregulated (green shaded areas) proteins. Cluster analysis of D/H ratios further revealed two subgroups of the 44 patients: a subgroup that had numerous upregulated proteins (34 patients) and a subgroup that had relatively more separate and distinct expression profiles (10 patients). These differences suggest that, for early diagnosis, multiple biomarkers may provide better prediction accuracy compared to individual biomarkers.

Next, a GO analysis of the quantitative proteomics results was performed to pinpoint proteins and processes underlying the changes in urinary proteomes observed in HCC patients (Figure 4). Interestingly, proteins overexpressed (D/H > 1.5, $p < 0.05$) in HCC patients in comparison with healthy controls were linked to signal peptides, response to wounding, acute inflammatory response, calcium ion binding, and α-amylase activity (Figure 4A). Figure 4B shows that downregulation of proteins was significantly correlated with microtubule motor activity with D/H < 0.5 and $p < 0.05$ and microtubule motor activity. The roles of signal peptides, cytokines, and secreted proteins have been studied extensively because of their important roles in the HCC tumor microenvironment [4]. Therefore, urinary peptides may reflect signals or signatures involved in cancer cell—host interaction and inflammatory process [52]. The criteria for further analysis of a "hit" were penetrance of at least 50% and a D/H value > 1.5; of the 25 proteins that met these criteria (Figure 4C), the top six were ROBO4, AXL, S100A9, TFF2, ARSA, and GRN (Figure 4D). In adults, ROBO4 expression is

Figure 3. Hierarchical clustering of proteins that showed significant ($p < 0.05$) differences in expression between urine samples from HCC patients and from healthy controls ($n = 44$). Wilcoxon signed-rank test with paired setting was used to calculate p values. Heat map was constructed by R package "pheatmap" (R Foundation foe Statistical Computing Vienna, Austria) with default settings. The value shown in the heatmap is the quantification ratio (D/H) of peptides identified in urine from HCC patients (deuterium labeling) to that in urine from normal controls (hydrogen labeling). HCC = hepatocellular carcinoma.

reportedly confined to vasculature and is overexpressed in tumor endothelial cells in comparison to normal endothelial cells [56]. Several candidates were related to HCC proliferation and invasion. The AXL promotes tumor invasion through transcriptional activation of Slug in HCC, and inhibiting AXL suppresses Slug expression and invasiveness in HCC cell lines [57].

Use of onco-proteogenomics for discovering HCC biomarkers

In recent years, HCC has been the most rapidly increasing cause of cancer-related mortality in Taiwan [11] and the United States (US) [58]. Because HCC is highly aggressive

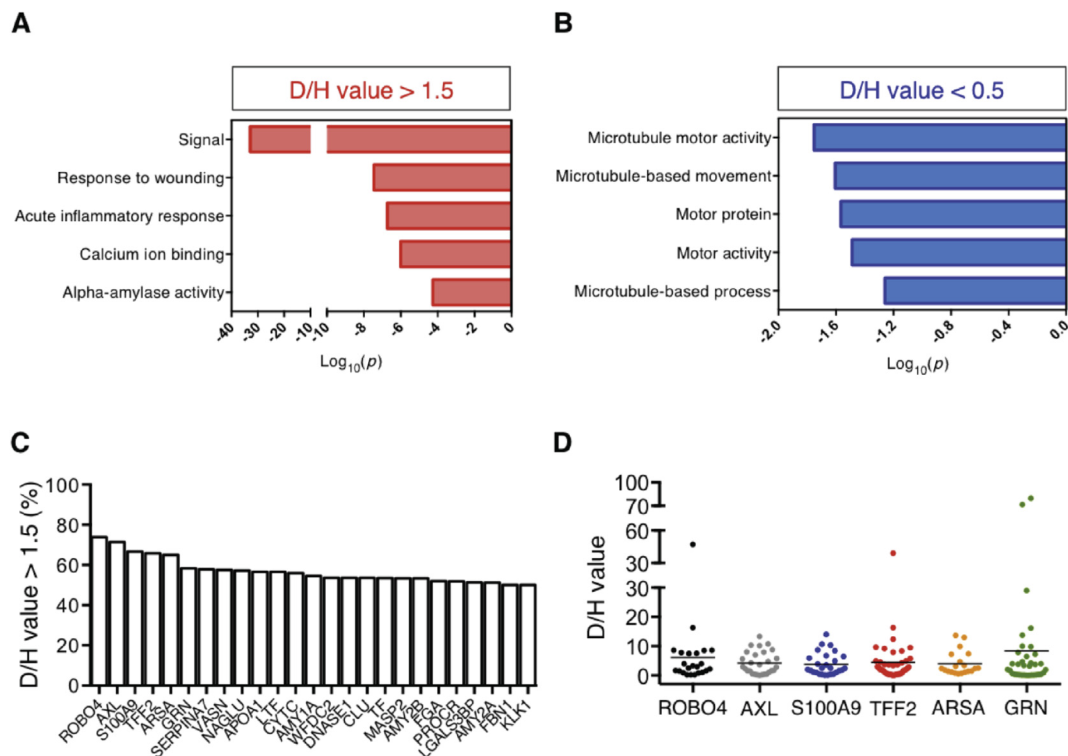


Figure 4. Identification of top upregulated proteins in urine samples from HCC patients ($n = 44$). Gene ontology analysis was used to classify major functional processes among (A) 82 upregulated (D/H > 1.5, $p < 0.05$) and (B) seven downregulated (D/H < 0.5, $p < 0.05$) proteins. (C) Upregulated proteins with at least 50% penetrance. The y axis is the frequency of upregulated proteins (D/H > 1.5) identified in urine samples from HCC patients. (D) D/H values of top six candidates were obtained and selected from individual sample pairs. HCC = hepatocellular carcinoma.

and is often detected at an advanced stage, most HCC patients are ineligible for transplantation or liver resection. Therefore, biomarkers for early detection and noninvasive diagnosis and prognosis of HCC are urgently needed.

The use of recently developed proteomic methodologies, for example, shotgun proteomics, for qualitative and quantitative analysis enable not only identification of protein mixtures in biological tissues with high reliability and high throughput, but also comprehensive global analysis of protein expression profiles in various cancer types, including HCC [20–23]. However, a remaining obstacle to using onco-proteomics to identify tumor-specific peptides is the complexities of human cancer genomes and transcriptomes, for example, point mutations, copy-number aberrations, or unusual gene-splicing variants [24]. Onco-proteogenomics is a new research strategy that solves this problem by integrating proteomics, genomics, and transcriptomics for detection of tumor-specific changes in the proteome. Thus, onco-proteogenomics enables in-depth understanding of the initiation and maintenance of tumors as well as their responses to drug treatments. Notably, the United States National Cancer Institute has established a proteogenomics program that integrates cancer genomics and proteomics [59]. Combining MS-generated data with genomic and transcriptomic information, reveals how oncogenic signaling pathways are dysregulated in various cancer types, including colorectal and gastric cancer [30–32].

Because onco-proteogenomics technology enables large-scale systematic monitoring of HCC genomes and corresponding protein variations in the translational landscape of cancer cells, onco-proteogenomics is an ideal tool for identifying potential biomarkers for early diagnosis of HCC. Notably, researchers have focused on identifying biomarkers in urine, which is stable compared to other body fluids and can be obtained noninvasively in large quantities. Figure 4 and Table 1 show that the integration of proteomic, genomic, and transcriptomic analyses in the proposed proteogenomic approach revealed significantly higher co-amplification of S100A9 and GRN ($p < 0.001$) and

Table 1 Proteomic analysis identifies potential biomarkers for HCC.

Cancer	Method	Source	Identified biomarkers	Refs
HCC	Proteogenomics (shotgun proteomics and stable isotope dimethyl labeling)	Urine from HCC patients and healthy controls	Urinary S100A9 and GRN	Huang et al, 2015 [55]

HCC = hepatocellular carcinoma.

significantly higher co-expression of S100A9 and GRN ($p < 0.01$) in HCC tumors and urine samples compared to controls. Notably, co-amplification of S100A9 and GRN was also associated with poor survival in HCC patients. Overall, our data suggest that S100A9 and GRN in urine have potential use as combinatorial biomarkers for early diagnosis of HCC. Our data also indicate the feasibility of using oncoproteogenomics to elucidate the complex relationships among proteomes and to identify protein biomarkers applicable in patient-specific translational medicine, that is, personalized translational therapy.

Conclusion and future perspectives

Due to rapidly improving algorithms and software for shotgun proteomic data analysis, the gel-free shotgun approach to quantitative proteomics is now as accurate as conventional shotgun genomic approaches. Given its advantages, the gel-free shotgun approach should be considered as the method of choice and preferred methodology for elucidating the complex relationships between human proteomes and various genome-related diseases. Methods currently used for diagnosis and prognosis of HCC are generally unsatisfactory because HCC is difficult to diagnose at an early stage, has a high recurrence rate after surgery, and is resistant to chemotherapy. The aims of this study were to provide an overview of newly developed platforms for using highly sensitive noninvasive diagnostic tools for profiling protein expression and to evaluate the use of shotgun proteomics coupled with an integrated oncoproteogenomics methodology, to improve understanding of HCC carcinogenesis and progression and to discover novel diagnostic and therapeutic methods. The focus of this study was identifying and characterizing HCC biomarkers. By examining and analyzing protein expression profiles in urine and tissues from HCC patients and normal controls, we have already positively identified two inflammation mediators, that is, S100A9 and GRN. These protein biomarkers in the alarmin family are found in most cells, especially immune cells such as monocytes and macrophages. This group of inducible endogenous cellular proteins or peptides is secreted from cells upon infection or inflammation and has a vital regulatory role in the host innate immune system [59–70]. Thus, the HCC-associated cancer-specific biomarkers described herein may contribute to dysregulatory processes associated with inflammatory and autoimmune conditions, as well as tumorigenesis and cancer metastasis. Moreover, they have potential use as combinatorial biomarkers for early diagnosis in personalized precision medicine.

Acknowledgments

This work was supported in part by the Kaohsiung Medical University for the program of "Aim for the Top Universities Grants" (Grant Nos. KMU-TP103E15 and KMU-TP104E20 to S.-H.C.), the National Science Council (NSC Grant 102-2314-B-037-037-MY2 to S.-H.C. and K.-T.L.), Ministry of Science and Technology, Taipei, Taiwan (Grant Nos. MOST 103-2320-B-037-021, MOST 104-2320-B-037-022, and MOST 104-2627-M-037-002 to S.-H.C.) and the Taiwan Protein Project of Academia Sinica, funded by Ministry of Science and Technology (MOST105-0210-01-12-01). We also thank the technical support of Drs. C.-H. Huang and P.-J. Pai concerning data analysis and manuscript preparation. The core facility grant (NSC Grant 99-2745-B-037-005) of the Quantitative Proteomics Center at the Center for Research Resources and Development (CRRD), Kaohsiung Medical University was under the auspices of National Science Council.

References

[1] Aebersold R, Mann M. Mass spectrometry-based proteomics. Nature 2003;422:198–207.
[2] Fehniger TE, Marko-Varga G. Proteomics and disease revisited: the challenge of providing proteomic tools into clinical practice. J Proteome Res 2010;9:1191–2.
[3] Boja E, Hiltke T, Rivers R, Kinsinger C, Rahbar A, Mesri M, et al. Evolution of clinical proteomics and its role in medicine. J Proteome Res 2011;10:66–84.
[4] Leonardi GC, Candido S, Cervello M, Nicolosi D, Raiti F, Travali S, et al. The tumor microenvironment in hepatocellular carcinoma (review). Int J Oncol 2012;40:1733–47.
[5] Chiou SH, Wu CY. Clinical proteomics: current status, challenges, and future perspectives. Kaohsiung J Med Sci 2011;27:1–14.
[6] Ansong C, Purvine SO, Adkins JN, Lipton MS, Smith RD. Proteogenomics: needs and roles to be filled by proteomics in genome annotation. Brief Funct Genomic Proteomic 2008;7:50–62.
[7] Armengaud J. Proteogenomics and systems biology: quest for the ultimate missing parts. Expert Rev Proteomics 2010;7:65–77.
[8] Mann M, Kulak NA, Nagaraj N, Cox J. The coming age of complete, accurate, and ubiquitous proteomes. Mol Cell 2013;49:583–90.
[9] Renuse S, Chaerkady R, Pandey A. Proteogenomics. Proteomics 2011;11:620–30.
[10] Villanueva A, Hernandez-Gea V, Llovet JM. Medical therapies for hepatocellular carcinoma: a critical view of the evidence. Nat Rev Gastroenterol Hepatol 2013;10:34–42.
[11] Kee KM, Lu SN. Hospital- and community-based screenings for hepatocellular carcinoma in Taiwan. Oncology 2011;81(Suppl. 1):36–40.
[12] Farazi PA, DePinho RA. Hepatocellular carcinoma pathogenesis: from genes to environment. Nat Rev Cancer 2006;6:674–87.
[13] Sanyal AJ, Yoon SK, Lencioni R. The etiology of hepatocellular carcinoma and consequences for treatment. Oncologist 2010; 15(Suppl. 4):14–22.
[14] Llovet JM, Ricci S, Mazzaferro V, Hilgard P, Gane E, Blanc JF, et al. Sorafenib in advanced hepatocellular carcinoma. N Engl J Med 2008;359:378–90.
[15] Huang CH, Lujambio A, Zuber J, Tschaharganeh DF, Doran MG, Evans MJ, et al. CDK9-mediated transcription elongation is required for MYC addiction in hepatocellular carcinoma. Genes Dev 2014;28:1800–14.
[16] Goyal L, Muzumdar MD, Zhu AX. Targeting the HGF/c-MET pathway in hepatocellular carcinoma. Clin Cancer Res 2013; 19:2310–8.
[17] Yamao T, Kai S, Kazami A, Koizumi K, Handa T, Takemoto N, et al. Tumor markers CEA, CA19-9 and CA125 in monitoring of response to systemic chemotherapy in patients with advanced gastric cancer. Jpn J Clin Oncol 1999;29:550–5.
[18] Zhang B, Yang B. Combined alpha fetoprotein testing and ultrasonography as a screening test for primary liver cancer. J Med Screen 1999;6:108–10.
[19] Chiou SH, Huang CH, Liang SS. From chemistry to translational medicine: the application of proteomics to cancer biomarker discovery and diagnosis. J Chin Chem Soc Taip 2015;62:217–26.

[20] Ideker T, Thorsson V, Ranish JA, Christmas R, Buhler J, Eng JK, et al. Integrated genomic and proteomic analyses of a systematically perturbed metabolic network. Science 2001;292:929—34.
[21] Washburn MP, Wolters D, Yates 3rd JR. Large-scale analysis of the yeast proteome by multidimensional protein identification technology. Nat Biotechnol 2001;19:242—7.
[22] McCormack AL, Schieltz DM, Goode B, Yang S, Barnes G, Drubin D, et al. Direct analysis and identification of proteins in mixtures by LC/MS/MS and database searching at the low-femtomole level. Anal Chem 1997;69:767—76.
[23] MacCoss MJ, McDonald WH, Saraf A, Sadygov R, Clark JM, Tasto JJ, et al. Shotgun identification of protein modifications from protein complexes and lens tissue. Proc Natl Acad Sci U S A 2002;99:7900—5.
[24] Alfaro JA, Sinha A, Kislinger T, Boutros PC. Onco-proteogenomics: cancer proteomics joins forces with genomics. Nat Methods 2014;11:1107—13.
[25] Nesvizhskii AI. Proteogenomics: concepts, applications and computational strategies. Nat Methods 2014;11:1114—25.
[26] Laurent-Puig P, Legoix P, Bluteau O, Belghiti J, Franco D, Binot F, et al. Genetic alterations associated with hepatocellular carcinomas define distinct pathways of hepatocarcinogenesis. Gastroenterology 2001;120:1763—73.
[27] Okamoto H, Yasui K, Zhao C, Arii S, Inazawa J. PTK2 and EIF3S3 genes may be amplification targets at 8q23-q24 and are associated with large hepatocellular carcinomas. Hepatology 2003;38:1242—9.
[28] Sawey ET, Chanrion M, Cai C, Wu G, Zhang J, Zender L, et al. Identification of a therapeutic strategy targeting amplified FGF19 in liver cancer by Oncogenomic screening. Cancer Cell 2011;19:347—58.
[29] Hoshida Y, Nijman SM, Kobayashi M, Chan JA, Brunet JP, Chiang DY, et al. Integrative transcriptome analysis reveals common molecular subclasses of human hepatocellular carcinoma. Cancer Res 2009;69:7385—92.
[30] Wang X, Slebos RJ, Wang D, Halvey PJ, Tabb DL, Liebler DC, et al. Protein identification using customized protein sequence databases derived from RNA-Seq data. J Proteome Res 2012;11:1009—17.
[31] Halvey PJ, Wang X, Wang J, Bhat AA, Dhawan P, Li M, et al. Proteogenomic analysis reveals unanticipated adaptations of colorectal tumor cells to deficiencies in DNA mismatch repair. Cancer Res 2014;74:387—97.
[32] Aquino PF, Lima DB, de Saldanha da Gama Fischer J, Melani RD, Nogueira FC, Chalub SR, et al. Exploring the proteomic landscape of a gastric cancer biopsy with the shotgun imaging analyzer. J Proteome Res 2014;13:314—20.
[33] Kuo CJ, Liang SS, Hsi E, Chiou SH, Lin SD. Quantitative proteomics analysis of varicose veins: identification of a set of differentially expressed proteins related to ATP generation and utilization. Kaohsiung J Med Sci 2013;29:594—605.
[34] Chiou SH, Lee MY, Kuo CJ, Liang SS. Application of quantitative proteomics to disease diagnosis and prognosis using stable isotope dimethyl labeling coupled with shotgun proteomics strategy and multiple reaction monitoring. In: Li MD, editor. Recent Advances in Proteomics Research. New York, U.S.A.: Nova Science Publishers; 2013. p. 119—37.
[35] Liang SS, Kuo CJ, Chi SW, Wu WJ, Chen ST, Chiou SH. Quantitative proteomics analysis of differentially expressed proteins involved in renal clear cell carcinoma by shotgun approach coupled with stable isotope dimethyl labeling. J Proteomics Bioinform 2013;S7:003.
[36] Kuo CJ, Wang SN, Liang SS, Chi SW, Yu ZJ, Hsi E, et al. Shotgun proteomics analysis of differentially expressed urinary proteins involved in the hepatocellular carcinoma. J Proteomics Bioinform 2014;7:34—40.
[37] Lee MY, Huang CH, Kuo CJ, Lin CL, Lai WT, Chiou SH. Clinical proteomics identifies urinary CD14 as a potential biomarker for diagnosis of stable coronary artery disease. PLoS One 2015;10:e0117169.
[38] Kuo KK, Kuo CJ, Chiu CY, Liang SS, Huang CH, Chi SW, et al. Quantitative proteomic analysis of differentially expressed protein profiles involved in pancreatic ductal adenocarcinoma. Pancreas 2016;45:71—83.
[39] Sze SK, Ge Y, Oh H, McLafferty FW. Top-down mass spectrometry of a 29-kDa protein for characterization of any posttranslational modification to within one residue. Proc Natl Acad Sci U S A 2002;99:1774—9.
[40] Kelleher NL. Top-down proteomics. Anal Chem 2004;76:197a—203a.
[41] Bogdanov B, Smith RD. Proteomics by FTICR mass spectrometry: top down and bottom up. Mass Spectrom Rev 2005;24:168—200.
[42] Chait BT. Chemistry. Mass spectrometry: bottom-up or top-down? Science 2006;314:65—6.
[43] Kovanich D, Cappadona S, Raijmakers R, Mohammed S, Scholten A, Heck AJ. Applications of stable isotope dimethyl labeling in quantitative proteomics. Anal Bioanal Chem 2012;404:991—1009.
[44] Hsu JL, Huang SY, Chow NH, Chen SH. Stable-isotope dimethyl labeling for quantitative proteomics. Anal Chem 2003;75:6843—52.
[45] Boersema PJ, Raijmakers R, Lemeer S, Mohammed S, Heck AJ. Multiplex peptide stable isotope dimethyl labeling for quantitative proteomics. Nat Protoc 2009;4:484—94.
[46] Boersema PJ, Aye TT, van Veen TA, Heck AJ, Mohammed S. Triplex protein quantification based on stable isotope labeling by peptide dimethylation applied to cell and tissue lysates. Proteomics 2008;8:4624—32.
[47] DeSouza LV, Siu KW. Mass spectrometry-based quantification. Clin Biochem 2013;46:421—31.
[48] Ting L, Rad R, Gygi SP, Haas W. MS3 eliminates ratio distortion in isobaric multiplexed quantitative proteomics. Nat Methods 2011;8:937—40.
[49] Perkins DN, Pappin DJ, Creasy DM, Cottrell JS. Probability-based protein identification by searching sequence databases using mass spectrometry data. Electrophoresis 1999;20:3551—67.
[50] Hirosawa M, Hoshida M, Ishikawa M, Toya T. MASCOT: multiple alignment system for protein sequences based on three-way dynamic programming. Comput Appl Biosci 1993;9:161—7.
[51] Huang da W, Sherman BT, Lempicki RA. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. Nat Protoc 2009;4:44—57.
[52] Roessler S, Jia HL, Budhu A, Forgues M, Ye QH, Lee JS, et al. A unique metastasis gene signature enables prediction of tumor relapse in early-stage hepatocellular carcinoma patients. Cancer Res 2010;70:10202—12.
[53] Wu HC, Wang Q, Yang HI, Ahsan H, Tsai WY, Wang LY, et al. Urinary 15-F2t-isoprostane, aflatoxin B1 exposure and hepatitis B virus infection and hepatocellular carcinoma in Taiwan. Carcinogenesis 2008;29:971—6.
[54] Scaggiante B, Kazemi M, Pozzato G, Dapas B, Farra R, Grassi M, et al. Novel hepatocellular carcinoma molecules with prognostic and therapeutic potentials. World J Gastroenterol 2014;20:1268—88.
[55] Huang CH, Kuo CJ, Liang SS, Chi SW, Hsi E, Chen CC, et al. Onco-proteogenomics identifies urinary S100A9 and GRN as potential combinatorial biomarkers for early diagnosis of hepatocellular carcinoma. BBA Clin 2015;3:205—13.
[56] Avci ME, Konu O, Yagci T. Quantification of SLIT-ROBO transcripts in hepatocellular carcinoma reveals two groups of genes with coordinate expression. BMC cancer 2008;8:392.
[57] Lee HJ, Jeng YM, Chen YL, Chung L, Yuan RH. Gas6/Axl pathway promotes tumor invasion through the transcriptional activation of Slug in hepatocellular carcinoma. Carcinogenesis 2014;35:769—75.

[58] El-Serag HB. Hepatocellular carcinoma. N Engl J Med 2011; 365:1118—27.
[59] Rivers RC, Kinsinger C, Boja ES, Hiltke T, Mesri M, Rodriguez H. Linking cancer genome to proteome: NCI's investment into proteogenomics. Proteomics 2014;14:2633—6.
[60] Nie Y, Yang D, Oppenheim JJ. Alarmins and antitumor immunity. Clin Ther 2016;38:1042—53.
[61] Arshad MI, Khan HA, Noel G, Piquet-Pellorce C, Samson M. Potential therapeutic aspects of alarmin cytokine interleukin 33 or its inhibitors in various diseases. Clin Ther 2016;38. 1000—16.e1.
[62] Villarreal DO, Siefert RJ, Weiner DB. Alarmin IL-33 elicits potent TB-specific cell-mediated responses. Hum Vaccin Immunother 2015;11:1954—60.
[63] Theoharides TC, Petra AI, Taracanova A, Panagiotidou S, Conti P. Targeting IL-33 in autoimmunity and inflammation. J Pharmacol Exp Ther 2015;354:24—31.
[64] Steichen AL, Simonson TJ, Salmon SL, Metzger DW, Mishra BB, Sharma J. Alarmin function of galectin-9 in murine respiratory tularemia. PLoS One 2015;10:e0123573.
[65] Galimberti D, Bonsi R, Fenoglio C, Serpente M, Cioffi SM, Fumagalli G, et al. Inflammatory molecules in frontotemporal dementia: cerebrospinal fluid signature of progranulin mutation carriers. Brain Behav Immun 2015;49:182—7.
[66] Li N, Yamasaki K, Saito R, Fukushi-Takahashi S, Shimada-Omori R, Asano M, et al. Alarmin function of cathelicidin antimicrobial peptide LL37 through IL-36gamma induction in human epidermal keratinocytes. J Immunol 2014;193: 5140—8.
[67] Cayrol C, Girard JP. IL-33: an alarmin cytokine with crucial roles in innate immunity, inflammation and allergy. Curr Opin Immunol 2014;31:31—7.
[68] Diener KR, Al-Dasooqi N, Lousberg EL, Hayball JD. The multifunctional alarmin HMGB1 with roles in the pathophysiology of sepsis and cancer. Immunol Cell Biol 2013;91: 443—50.
[69] Mishra BB, Li Q, Steichen AL, Binstock BJ, Metzger DW, Teale JM, et al. Galectin-3 functions as an alarmin: pathogenic role for sepsis development in murine respiratory tularemia. PLoS One 2013;8:e59616.
[70] Oppenheim JJ, Tewary P, de la Rosa G, Yang D. Alarmins initiate host defense. Adv Exp Med Biol 2007;601:185—94.