Biomarkers for Recurrence of Hepatocellular Carcinoma

8

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Contents

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Abstract

Hepatocellular carcinoma (HCC) is one of the deadliest cancers in the world, and the prognosis of HCC patients remains poor despite earlier diagnosis and treatment. Recurrence is the main cause of death in patients who received curative treatment for the primary cancer. Tumors arising within 2 years of treatment typically originate from the primary tumour. These recurrent tumours tend to be aggressive and result in high mortality rate for these patients. Hence there is a clinical need to develop prognostic biomarkers for prediction of early recurrence. Proteomics involves the unbiased study of global changes of protein expression in a high throughput manner. This allows for identification of biomarkers for prediction of HCC recurrence. In this review, we described the proteomics workflow and platforms used in the biomarker discovery for HCC recurrence. We also summarized the main findings of studies that utilized proteomics as the primary tool for biomarker identification in patient samples. Lastly, we discussed the possible improvements in study design and technologies to translate the results obtained from proteomic studies to clinical use.

Keywords

Hepatocellular carcinoma • Intrahepatic metastasis • Mass spectrometry • Proteomics • Recurrence

List of Abbreviations

2D-DIGE	2-Dimensional-difference gel electrophoresis
2-DE	2-Dimensional electrophoresis
adoMET	S-Adenosylmethionine
AMACR	Alpha-methylacyl-CoA racemase
cICAT	Cleavable isotope-coded affinity tags
emPAI	Exponentially modified protein abundance index
HCC	Hepatocellular carcinoma
IEF	Isoelectric focusing
IHC	Immunohistochemistry
iTRAQ	Isobaric tags for relative and absolute quantitation
LC	Liquid chromatography
m/z	Mass-to-charge ratio
MALDI	Matrix-assisted laser desorption/ionization
MS	Mass spectrometry
NDRG1	N-myc downstream-regulated gene 1
PAGE	Polyacrylamide gel electrophoresis
p*I*	Isoelectric point
qRT-PCR	Quantitative real-time-polymerase chain reaction
SDS	Sodium dodecyl sulfate
SELDI	Surface-enhanced laser desorption/ionization

TMA	Tissue microarray analysis
TOF	Time of flight

Key Facts of Hepatocellular Carcinoma Recurrence

- Up to 80% of all hepatocellular carcinoma patients who received curative treatment suffer from recurrence.
- Recurrence is broadly divided into two types: intrahepatic metastasis and multicentric occurrence.
- Intrahepatic metastasis is associated with early recurrence (within 2 years after treatment), poor overall survival, and aggressive metastatic nature of recurrent tumors.
- Clonal analysis suggests that most of the tumors in early recurrence originate from the primary tumor.
- There are no effective prophylactic treatments available to prevent recurrence.
- Proteomic analyses of primary tumors in early recurrent patients aim to identify biomarkers for prediction and prophylactic treatment of early recurrence.

Definitions of Words and Terms

2-Dimensional electrophoresis	This is a proteomic technique involving the separation of proteins based on two different properties. Typically, proteins are first separated by their isoelectric point, followed by separation according to molecular weight.
Biomarker	A biomarker is a biological molecule that is characteristic of disease states or processes in the body. The levels of biomarkers can be detected and measured in the body.
Hepatocellular carcinoma	Hepatocellular carcinoma is the most common form of liver cancer. This form of cancer arises from neoplastic changes to the hepatocytes.
Intrahepatic metastasis	Intrahepatic metastasis is the formation of recurrent tumors that originate from the primary tumor. This phenomenon is strongly associated with venous invasion of the primary tumor to the portal vein.
Liquid chromatography	Liquid chromatography separates analytes in a complex mixture based on the strength of interaction between the stationary phase in the

	analytical column and the analytes in the liquid mobile phase.
Mass spectrometry	Mass spectrometry is an analytical method that detects the analyte ion in the gaseous phase which is specific to the mass over charge ratio of the analyte. In proteomics, peptides are first ionized and then converted to gaseous phase ions. The identity of the peptide can be determined by comparing the mass spectrum generated by tandem mass spectrometry analysis with a reference database. Protein identification is inferred by detecting tryptic peptides that are unique for each protein.
Multicentric carcinogenesis	Multicentric carcinogenesis refers to the formation of recurrent tumors that have different clonal origins as the primary tumors. These tumors can be considered as new primary tumors, and they are believed to be formed due to the accumulation of genetic changes in the cirrhotic liver.
Proteomics	Proteomics is the large-scale study of proteins to characterize biological processes. Expression proteomics is commonly used in biomarker discovery studies, in which quantitative/semiquantitative information of protein expression levels are obtained and compared between different disease states.
Sensitivity	Sensitivity refers to the ability to correctly identify true positives in the population. In a diagnostic test for a disease, the test with high sensitivity would be able to identify most of the patients with the disease.
Sodium dodecyl sulfate-polyacrylamide gel electrophoresis	This involves the separation of proteins by their molecular weight in denaturing conditions. The proteins in the samples are first denatured and reduced by addition of sodium dodecyl sulfate, reducing agents and heating. Proteins in the samples are separated in the polyacrylamide gel under the influence of an electric field.
Specificity	Specificity refers to the ability to correctly identify true negatives in the population. In a diagnostic test for a disease, the test with high specificity would be able to correctly identify patients without the disease and minimize placing patients without disease into the disease group (false positives).

Introduction

HCC is the fifth most prevalent cancer in the world, with increasing incidence in both Western and Asian countries (Ferlay et al. 2015). Technological advances in imaging have enabled the early detection and diagnosis of HCC (Mancuso 2013). However, the prognosis of HCC patients remains poor. HCC is the third most frequent cause of cancer-related death, with up to 80% of patients suffering from recurrence after treatment with curative intent such as liver transplantation, resection, or ablation (Franssen et al. 2014; Lau and Lai 2008; Poon et al. 2000). Recurrence is the main cause of cancer-related death in patients amenable to curative treatment. Hence, there is the clinical need to identify patients with higher risk of recurrence so that they could benefit from increased surveillance. These proteins might also be potential drug targets for development of novel prophylactic treatment to prevent recurrence.

HCC recurrence can arise from the undetected microscopic spread of the primary tumor (intrahepatic metastasis) or de novo formation of new tumors on the cirrhotic liver which provides a favorable "field" for accumulation of genetic alterations, eventually leading to neoplasia (multicentric carcinogenesis) (Sherman 2008). The risk factors and outcomes for these two types of recurrence are vastly different (Cheng et al. 2015; Du et al. 2014; Portolani et al. 2006; Wu et al. 2009). Intrahepatic metastasis usually occurs within 2 years of initial treatment. The early recurrent tumors are usually aggressive and refractory to subsequent treatment, resulting in a lower 5-year survival rate compared to patients with late recurrence. Risk factors for early recurrence are tumor related, of which venous invasion is the most common factor. Clonality analysis demonstrated that the recurrent tumors originate from the primary HCC (Morimoto et al. 2003; Wang et al. 2013; Zhang et al. 2015). Multicentric carcinogenesis occurs more than 2 years after treatment (late recurrence). The recurrent tumors are of different clonal origin; hence, they can be considered as new primary tumors. Most patients with multicentric carcinogenesis are suitable for treatment, and these patients have a comparable survival rate to that of the cirrhotic patients with a higher risk of developing primary HCC (Cucchetti et al. 2009). The risk factors for late recurrence are related to host factors such as degree of cirrhosis and function of remnant liver.

The poor prognosis of early recurrence and its correlation with the primary HCC led to studies on the primary tumor to decipher the molecular basis of recurrence and determine biomarkers that can predict the risk of early recurrence. Proteomics provides a platform for large-scale studies of the proteome, in which differentially expressed proteins can be used as biomarkers to segregate patients according to risk of early recurrence. Furthermore, the expression patterns of the proteins allow for identification of molecular pathways that are dysregulated in the tumors of early recurrence patients, thus allowing for development of novel therapeutics that target key players involved in activation/inactivation of the pathways. In this review, we will outline the typical proteomic workflow in identification of potential prognostic biomarkers for HCC recurrence. Different techniques used in the separation and

quantitation of proteins/peptides will be discussed. In addition, we will summarize the findings from studies that utilized proteomics in the identification of biomarkers for early recurrence. Lastly, we will discuss the improvements that can be made to translate results generated from proteomics to clinical use.

Proteomics as a Tool for Discovery of Cancer Biomarkers

The typical workflow in proteomic identification of biomarkers is illustrated in Fig. 1. Most studies used liver tissues from the tumor and the adjacent non-tumor regions for proteomic-based discovery studies. Other possible samples include biological fluids such as serum/plasma, as they may contain proteins that are shred or actively secreted by the tumors into the blood circulation. These samples are then processed prior to separation, quantitation, and identification of proteins or peptides by proteomics. Proteomic technologies used in the separation and quantitation can be broadly divided into gel-based, liquid chromatography (LC)-based, and chip-based methods. Identification of proteins is usually achieved by tandem mass spectrometry (MS/MS) analysis of tryptic peptides. Finally, validation is performed to confirm the differential expression of proteins identified in the discovery phase in a separate cohort of clinical samples.

Validation is usually performed using antibody-based methods, such as Western blotting, immunohistochemistry (IHC), and tissue microarray analysis (TMA). Results from these assays would be analyzed in conjunction with clinical information of the patients to identify correlations of protein expression with prognosis of HCC. Functional studies can be performed on cell lines or animal models to correlate the changes in protein expression levels with different traits leading to cancer recurrence, thus providing an understanding of the biological pathways involved in HCC recurrence. After the clinical relevance of target protein has been established, the focus would be shifted to the development of a detection platform that allows for sensitive and reproducible analysis of target protein expression in the clinic. The following sections describe the different approaches that were applied in published studies that involved identification of HCC recurrence biomarkers, with emphasis on the proteins' separation and quantitation.

Gel-Based Proteomic Approaches

Gel-based proteomics involves the separation of proteins present in a complex sample by the polyacrylamide gel. 2-Dimensional electrophoresis (2-DE) is the main workhorse of gel-based proteomics, in which proteins are first separated by their isoelectric point (p*I*) in isoelectric focusing (IEF), followed by molecular size via sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The gels are subsequently stained to visualize the protein spots. Relative quantitation of

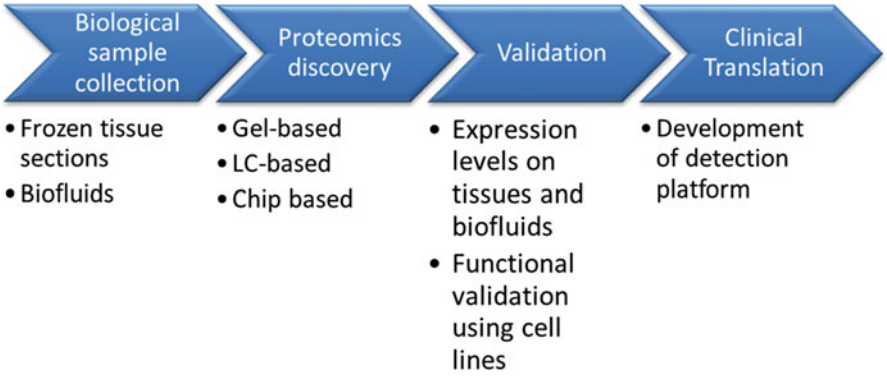

Fig. 1 Proteomic workflow for biomarker discovery. The typical proteomic workflow involves collection of biological samples that are relevant to the research question. Samples are then processed for application on the proteomic platform. Validation of protein expression and function is carried out to confirm the findings observed in the proteomic study and to provide evidence to the functional relevance of protein dysregulation in the disease of interest. After clinical validation, the focus will be shifted to the development of a suitable platform that would allow for detection of target proteins with high accuracy and reproducibility in the clinical setting

protein spots is achieved by comparing the intensity of protein spots with matched p*I* and molecular size in different gels.

Conventional 2-DE suffers from lack of reproducibility of the spot patterns and intensities due to the high levels of inherent technical and systematic variability (Voss and Haberl 2000). This results in the difficulty to distinguish true biological variations from technical artifacts. 2-Dimensional-difference gel electrophoresis (2D-DIGE) was introduced by Minden's lab to minimize technical variations associated with conventional 2-DE (Unlu et al. 1997). This decrease in variation is brought about by the simultaneous separation of two different samples on a single 2-DE experiment. In addition, an additional sample can be labeled by Cy2, which can serve as an internal standard to remove technical variations arising from individual 2-DE experiments during data analysis. The workflow for 2D-DIGE is illustrated in Fig. 2. Readers are recommended to refer to a recent review (Arentz et al. 2015) for a comprehensive understanding of the technical considerations in the design of 2D-DIGE experiments.

Liquid Chromatography-Based Proteomic Approaches

LC-based proteomics involves the tryptic digestion of proteins into peptides, before separation of peptides by LC to reduce the complexity of the samples. The mode of separation depends on the chemistry of the column, among which strong cation exchange and reverse phase columns are commonly used. These columns separate

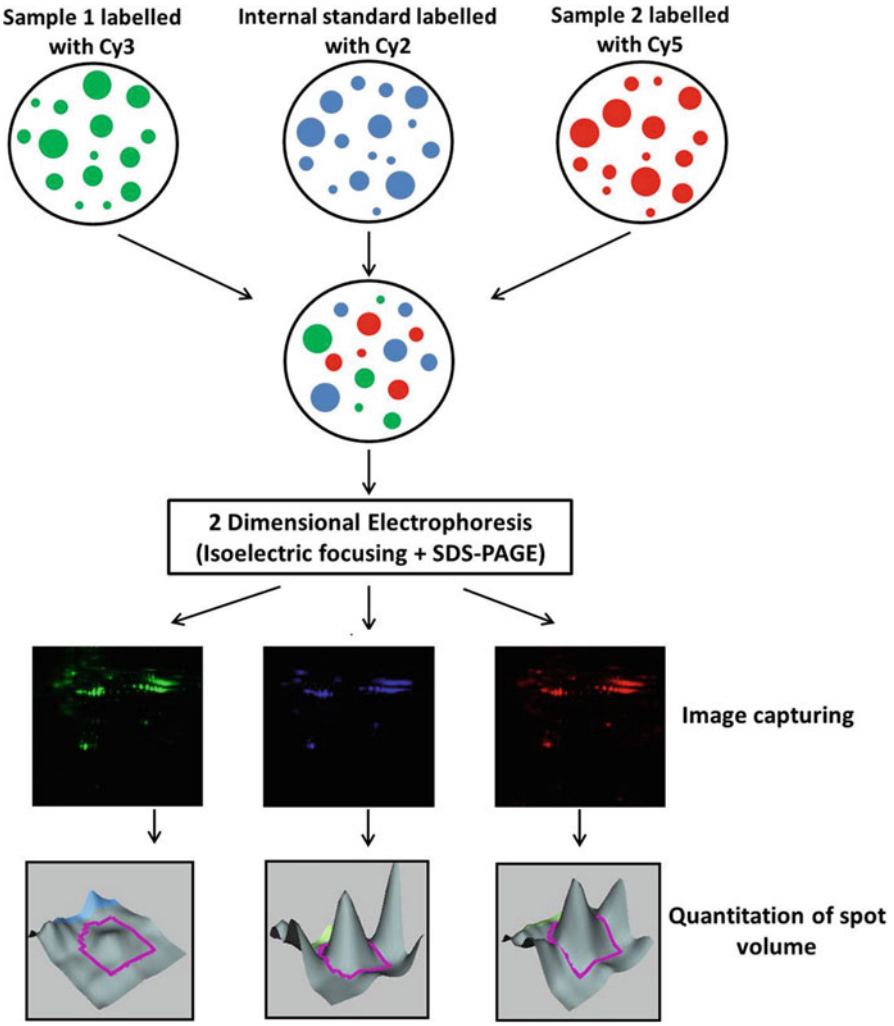

Fig. 2 2D-DIGE workflow. In 2D-DIGE, two different protein samples are labeled with Cy3 and Cy5, respectively. An internal reference sample, which is usually formed by pooling equal amounts of all samples used in the DIGE experiment, is labeled with Cy2. These three samples are then mixed, before the proteins are subjected to separation by isoelectric focusing and gel electrophoresis. The protein maps of different samples are visualized and captured by excitation of the fluorophores on the CyDyes. The images are exported to software for processing and normalization procedures. Relative quantitation is obtained by comparing the volumes of matched protein spots in different samples

the peptides based on charge and hydrophobicity, respectively. Identification of proteins in the sample is achieved by MS/MS analysis of the peptides. Various methods of protein quantitation are available, including labeling (chemical labeling) and label-free methods (spectrum counting), which will be discussed in the following sections.

Chemical Labeling: Cleavable Isotope-Coded Affinity Tags (cICAT) and Isobaric Tags for Relative and Absolute Quantitation (iTRAQ)

The proteins in the samples can be labeled with chemical tags for quantitation. The labeling process involves the formation of a chemical bond between the tag and the protein/peptide, followed by mixing of labeled samples before LC separation and MS analysis. Examples of chemical labeling tags include cICAT (Gygi et al. 1999) and iTRAQ (Choe et al. 2007; Ross et al. 2004). cICAT and iTRAQ differ in the labeling and method of obtaining quantitative data from mass spectrometry. cICAT labels carry a sulfhydryl reactive group, linker region containing either light or heavy isotopes ($^{12}C/^{13}C$), and a biotin moiety. Protein samples are labeled and trypsinized, and the labeled peptides are purified by avidin affinity chromatography. Cleavage of biotin tag is required as the full tag is too big and interferes with ionization of the peptides. Peaks corresponding to the same peptide from the two different samples will appear as a doublet with a mass difference of 9 Da. Quantitative information can be obtained by comparing the peak intensity of the two distinct peaks in the MS spectrum.

In contrast, the proteins from the different samples are trypsinized before labeling with the iTRAQ labels. These labels contain an amine-reactive group, a balance group, and a reporter mass (114 to 117 for 4-plex, 113–121, excluding 120, for 8-plex). The different labels are isobaric and thus have the same mass and chemical properties. Samples are mixed after labeling and subsequently subjected to LC-MS analysis. The precursor ions from all the different samples will elute at the same time for MS analysis. Quantitative information is obtained in the MS/MS analysis, in which the reporter ions will be dissociated and their peak intensities can be compared for determination of relative abundance of proteins. Both cICAT and iTRAQ workflows involve mixing of labeled samples before LC-MS analysis, thus allowing for multiplexing of samples and avoiding technical variations arising from LC separation and MS analysis. The workflows for cICAT and iTRAQ are summarized in Fig. 3.

Label-Free Proteomic Approach

In a label-free proteomic approach, different samples are processed separately during LC-MS analysis. Spectrum counting is the most basic label-free quantitation method, which is based on the fact that an increased abundance of proteins will result in an increased number and frequency of peptides observed (Washburn et al. 2001). Spectrum counting methods can be used to determine the relative abundance of the protein by using exponentially modified protein abundance index (emPAI) (Ishihama et al. 2005). Recently, a novel MS strategy, termed SWATH-MS (Gillet et al. 2012), has been developed. SWATH-MS utilizes a sequential mass window to acquire MS/MS information of all detectable peptides within that mass window. The information acquired can be used to generate a complete fragment ion map of the proteome. Quantitative information can be obtained from the extracted

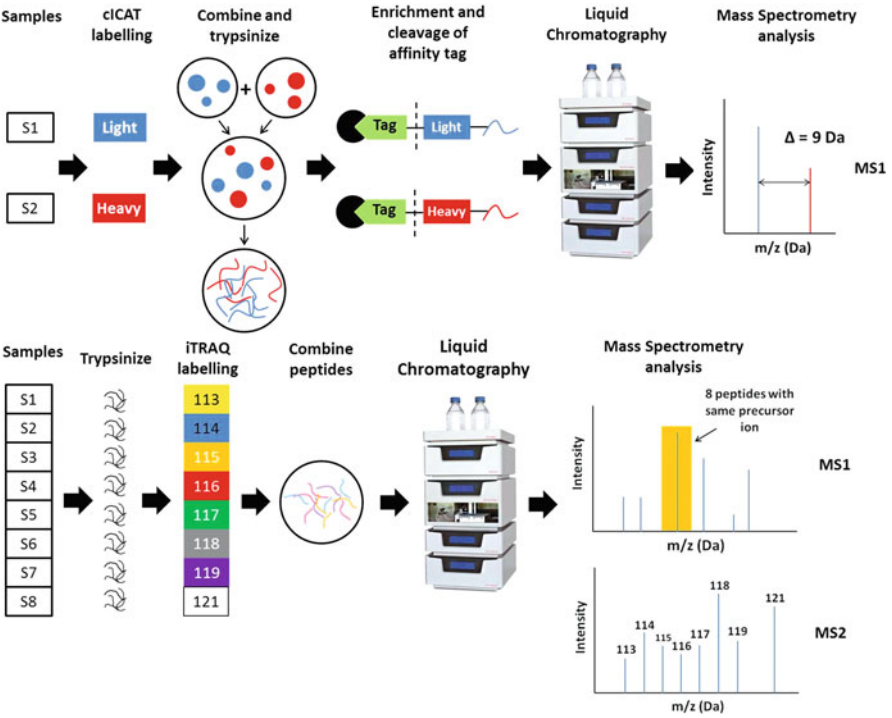

Fig. 3 Workflow of (**a**) cICAT and (**b**) iTRAQ analysis. Pictorial representation of the workflow of (**a**) cICAT and (**b**) iTRAQ analysis. (**a**) cICAT analysis involves the labeling of proteins of two different samples with cICAT labels containing either light or heavy isotopes of carbon. The two samples are then mixed, trypsin digested before enrichment of tagged peptides. The biotin tags on the labeled peptides are then cleaved before the peptides are subjected to liquid chromatography-mass spectrometry analysis. Peptides of the same protein will appear as a doublet with a mass difference of 9 Da in mass spectrometry analysis (MS1). (**b**) iTRAQ analysis involves trypsinization of individual samples, before labeling of up to eight different samples with the iTRAQ reagents. The labeled peptides are combined, before subjected to liquid chromatography-tandem mass spectrometry analysis. Quantitative information is obtained by comparing the relative intensity of the reporter ions after tandem mass spectrometry analysis (MS2)

ion chromatogram of the peptides. SWATH-MS strategy allows for digital archiving of tumor proteome, allowing retrospective quantitative comparison of targeted proteins in different samples when new research questions arise.

Chip-Based Proteomics: Surface-Enhanced Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (SELDI-TOF-MS)

SELDI-TOF-MS combines retentate chromatography and mass spectrometry on a chip to identify biomarkers for analytical purposes (Issaq et al. 2002). These chips contain ligands that have been derivatized with different chromatographic

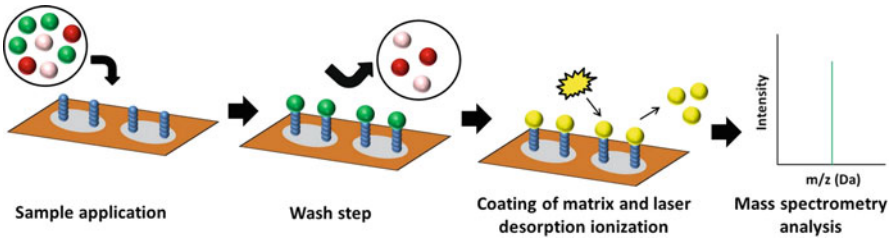

Fig. 4 SELDI-TOF-MS workflow. Samples are applied to ProteinChips, which are derivatized with chromatography ligands. Some of the chromatographic chemistries available include ion exchange, reverse phase, normal phase, and immobilized metal affinity chromatography. The unbound proteins are then washed away. Subsequently, the proteins are coated with a layer of matrix, which is necessary for laser desorption ionization. Quantitative information is obtained by comparing intensities of the peaks with the same mass-to-charge ratios in different samples

chemistries. The procedure involves sample application, washing of unbound proteins, coating of matrix, and analysis of proteins present on chip by MS. Quantitative information is obtained by comparing the area under peaks with defined *m*/*z* values. One drawback of SELDI-TOF-MS is that the identity of the peptide that produces the particular peak with the *m*/*z* value is unknown, and further analysis is required to identify the protein of interest. The SELDI-TOF-MS workflow is summarized in Fig. 4.

Biomarkers of HCC Recurrence Identified from Proteomic Studies

For the selection of publications to be included in this review, the PubMed database was searched with the term "((Hepatocellular carcinoma) AND Recurrence) AND Proteomics)." We selected papers that utilized biological samples obtained from patients and the usage of proteomics as the primary tool for biomarker discovery. There are a total of ten publications that fulfilled this requirement. Nine of the publications utilized frozen tissue samples, while only one publication used serum for biomarker discovery. The details of the publications are summarized in Table 1. Most of the publications focused on the discovery of candidate biomarkers based on their discriminatory powers between patients with or without cancer recurrence. The other publications are based on their discovery or validation criteria of known tumor factors and pathways associated with poor prognosis of HCC. These publications would be discussed in greater detail in the subsequent sections.

Biomarker Discovery Based on Time to Recurrence

The first group of studies grouped the patients solely on the presence and absence of recurrence within a stipulated time period for the discovery studies. These studies choose protein(s) with high discriminatory power in the discovery set for subsequent

Table 1 Proteomic studies for identification of HCC recurrence biomarkers. This table summarizes the main findings of the proteomic studies that attempted to identify predictive biomarkers for HCC recurrence. The papers are ordered in chronological order. The regulations of the candidate proteins are denoted by the *arrows*, where ↑ indicates an upregulation and ↓ indicates a downregulation of protein expression in recurrence patients compared to non-recurrence

Reference	Sample type	Curative treatment	Classification	Etiology	Discovery platform	Candidate biomarkers (regulation)	Validation
Yokoo et al. (2007)	Tumor tissues	Surgical resection	Recurrence within 6 months post surgery versus no recurrence 2 years post surgery	Mixed	2D-DIGE, MALDI-TOF/MS	Panel of 23 proteins (10 ↑ 13↓)	2D-DIGE
Orimo et al. (2008)	Tumor and non-tumor tissues	NA	Tissue differentiation	Mixed, mostly HCV	2D-DIGE, LC-MS/MS	Microtubule-associated protein RP/EB family member 1 (↑)	IHC
Yi et al. (2008)	Tumor, non-tumor, and normal liver tissues	Surgical resection	Recurrence versus no recurrence within 1 year post surgery	HBV	2-DE, MALDI-TOF/MS	Stress-70 protein, mitochondrial (↑)	IHC
Bai et al. (2009)	Tumor and non-tumor tissues	Liver transplantation	Recurrence versus no recurrence within 3 years post transplantation	HBV	cICAT, 2DLC-MS/MS	Calpain small subunit 1 (↓)	qRT-PCR, Western blot, IHC
Cheng et al. (2011)	Tumor tissues	Liver transplantation	Recurrence versus no recurrence (time frame not stated)	Not stated	2-DE, MALDI-TOF/MS	Protein NDRG1 (↑)	Western blot, IHC
Kanamori et al. (2011)	Tumor and non-tumor tissues	NA	Tumor versus adjacent non-tumor	HCV	2DLC-MS/MS	Talin-1 (↑)	IHC

Cao et al. 2013	Serum	Microwave ablation	Recurrence versus no recurrence within 1 year post curative MWA	HBV	SELDI-TOF-MS	m/z of 7,787, 6,858 and 6,646 (↓)	SELDI-TOF-MS
Tan et al. (2014)	Paired tumor and non-tumor tissues	Surgical resection	Recurrence versus no recurrence within 2 years post surgery	Mostly HBV	2D-DIGE, MALDI-TOF/MS		IHC, TMA
Taoka et al. (2014)	Tumor, non-tumor, and normal liver tissues	Surgical resection	Recurrence within 2 years (ER), no recurrence after 2 years (LR), adjacent non-tumor tissue, or normal liver tissue	Mixed	PROTOMAP profiling method (SDS-PAGE, LC-MS/MS)	Signal transducer and activator of transcription 1-alpha/beta (↑)	Western blot
Huang et al. (2014)	Tumor tissues	Surgical resection	Recurrence within 6 months, recurrence between 6 and 12 months, and no recurrence within 2 years post surgery	HBV	iTRAQ-2DLC-MS/MS	Alpha-methylacyl-CoA racemase (↓) Protein S100-A12 (↓)	IHC

HBV hepatitis B virus, *HCV* hepatitis C virus, *2D-DIGE* 2-dimensional-difference gel electrophoresis, *MALDI-TOF/MS* matrix-assisted laser desorption ionization time of flight, *LC-MS/MS* liquid chromatography-tandem mass spectrometry, *cICAT* cleavable isotope-coded affinity tags, *iTRAQ* isobaric tags for relative and absolute quantitation, *IHC* immunohistochemistry, *qRT-PCR* quantitative real-time-polymerase chain reaction, *SELDI-TOF-MS* surface-enhanced laser desorption/ionization time-of-flight mass spectrometry, *TMA* tissue microarray

clinical validation. Yi et al. applied conventional 2-DE on tissue lysates of matched tumor and noncancerous tissues of hepatitis B virus (HBV)-positive patients for discovery of recurrence-related biomarkers (Yi et al. 2008). Stress-70 protein was determined to have the highest discriminatory capacity (sensitivity = 90.9%, specificity = 71.4%) in distinguishing patients with early recurrence within 1 year of surgery. Its expression was positively correlated to increasing metastatic capacity in HCC cell lines and associated with advanced tumor stage and positive venous invasion. Huang et al. used iTRAQ-based LC-MS/MS approach to interrogate the tumor proteome of HCC patients with no recurrence, early recurrence, and late recurrence (Huang et al. 2014). Alpha-methylacyl-CoA racemase (AMACR) and S100-A12 were found to be reversely regulated in patients with early and late recurrence, of which both proteins were downregulated in early recurrence and upregulated in late recurrence with respect to patients with no recurrence. Low AMACR expression was subsequently determined to be correlated with poor prognosis of HCC patients by tissue microarray analysis (Xu et al. 2014).

Some studies might propose algorithms for prediction of recurrence risk based on data from the discovery set, and the performance of these algorithms would be validated on a separate cohort. This approach was employed by Yookoo et al., who applied 2D-DIGE analysis of tumor tissue lysates to determine proteins that can separate patients with different recurrence status (Yokoo et al. 2007). A panel of 23 protein spots was identified, and this panel successfully grouped 16 out of 17 patients in validation cohort according to their recurrence status. However, the validation method involves the use of 2D-DIGE to obtain the proteome map. This requires high level of technical competence; hence, it may be challenging to translate the above results into the clinic. Similarly, Tan et al. applied 2D-DIGE to identify biomarkers that can distinguish between stage I patients with early recurrence leading to aggressive metastatic HCC and patients with no recurrence (Tan et al. 2014). Eight proteins were found to be differentially expressed, of which the expression of three proteins, namely, heat shock protein 70 kDa protein 1A/1B, argininosuccinate synthase, and UTP-glucose-1-phosphate uridylyltransferase, was validated using tissue microarray. An algorithm was generated based on the data obtained from tissue microarray for prediction of risk of aggressive metastatic recurrence. In another study, serum samples of patients who undergo microwave ablation for therapy were used for prediction of early HCC recurrence (Cao et al. 2013). The SELDI-TOF-MS platform was used, and nine different protein peaks were significantly regulated. Three protein peaks with m/z values of 7,787, 6,858, and 6,646 were used to generate a decision tree which has a sensitivity of 85.7% and specificity of 88.9%. The ease of translation combined with the use of minimally invasive biological fluid makes the use of SELDI-TOF-MS an attractive method for biomarker discovery. However, the lack of information on the proteins associated with the differentially regulated peaks makes it difficult for further functional studies to be performed. Hence, caution must be taken when using SELDI-TOF-MS as the discovery platform, as limited information can be obtained from the study.

Integration of Cancer-Associated Factors in the Study Design of Proteomic Studies

Some of the studies choose proteins for functional and clinical validation based on their biological relevance to recurrence or cancer progression. Two of the studies that identified protein biomarkers associated with early recurrence after liver transplantation took this approach. Bai et al. used cICAT LC-MS/MS to quantitate relative protein expression changes between these early recurrence and non-recurrence patients (Bai et al. 2009). Calpain small subunit 1 (Capn4) was selected for further functional validation based on its interaction with metastasis-related proteins, as well as the correlation of Capn4 expression to HCC cell lines with different metastatic potential. Knockdown of Capn4 resulted in decreased wound healing, migration, and invasion of highly metastatic MHCC97H cell line. High expression of Capn4 is associated with decreased survival rate and tumor factors associated with metastasis, such as the presence of venous invasion and the absence of tumor encapsulation. In another study, Cheng et al. used 2-DE to interrogate the tumor proteome of HCC patients with and without early recurrence (Cheng et al. 2011). N-myc downstream-regulated gene 1 protein (NDRG1) was selected for further validation as it was reported to be a downstream target of hypoxia-inducible factor 1α. Upregulation of NDRG1 in tumor tissues was validated via Western blot, and siRNA knockdown of NDRG1 resulted in decreased proliferation, migration, and invasion of HepG2 cells. High NDRG1 expression is also associated with poor prognosis and tumor factors such as increased tumor size and the presence of vascular invasion. These two studies had demonstrated the involvement of the proteins associated with HCC recurrence in metastasis-related processes and could be targets for prophylactic treatment.

In the other studies, the samples were selected based on pathological factors associated with HCC progression and prognosis. Orimo et al. used tumor tissues with different histological differentiation status and compared their proteomes to adjacent non-tumor tissues and normal liver tissues by 2D-DIGE (Orimo et al. 2008). Twenty-six differentially expressed proteins successfully segregated the patients according to the differentiation status (Orimo et al. 2008). Microtubule-associated protein RP/EB family member 1 (EB1) was selected for further validation based on its functional association with c-Myc, RhoA, and cdc42 and its high expression in poorly differentiated tumors. EB1 expression was subsequently determined to be an independent prognostic factor for recurrence and survival of patients after resection. Kanamori et al. analyzed four early HCC and non-HCC tissues from two patient cases to identify proteins involved in HCC progression by label-free LC-MS analysis (Kanamori et al. 2011). Sixty-one differentially regulated proteins were found, of which talin-1 was chosen for validation as many cytoskeletal proteins were reported to be associated with HCC progression. They showed that high talin-1 expression was associated with poor differentiation, the presence of portal vein invasion, and intrahepatic metastasis.

Lastly, the understanding of biological processes involved in HCC progression could affect the proteomic tool used in the study. Excessive proteolysis is observed

in pathological events such as cancer (van Kempen et al. 2006). Levels of aberrant proteolytic fragments may be correlated to the severity of the disease. As such, Taoka et al. attempted to use the PROTOMAP profiling approach to identify proteins with differential expression levels of normal and proteolytic fragments in patients with different recurrence status (Taoka et al. 2014). This approach involves the separation of tissue lysates by molecular weight via SDS-PAGE, cutting of gel pieces according to molecular weight, in-gel digestion, and LC-MS identification of the proteins. In addition, the protein abundance is estimated by emPAI parameters, which is subsequently used to generate heat map "peptographs." In these peptographs, the identified peptides for a given protein were plotted vertically (from N- to C-terminal), and the SDS migration of the protein and its fragments were plotted horizontally. The abundance of the peptide was indicated by the intensity of the heat map in the peptographs. Forty-six upregulated proteins and 41 downregulated proteins were identified, of which Western blot analysis clearly demonstrated the specific proteolysis of signal transducer and activator of transcription 1 (STAT1) in tumor tissue lysates of HCC patients with early recurrence.

Protein Biomarkers that Demonstrated Consistent Regulation in Multiple Publications

Typically, proteomic studies identified many proteins that were dysregulated, but only a minority were selected for validation due to limited amounts of biological samples, availability and cost of commercial antibodies for validation, and lack of novelty and knowledge of the biological function of these proteins. There are proteins that have been "validated" by being consistently found to be dysregulated in various studies and thus may be considered as potential markers. The list of proteins that were consistently regulated in two or more publications is shown in Table 2. Interestingly, several cytoskeletal proteins, heat shock proteins, and transcription regulators are upregulated in HCC recurrence. This could be linked to morphological changes, increased proliferation, and survival of tumor cells, leading to poor prognosis.

The liver is one of the main metabolic organs in the body; thus, it is not surprising that dysregulated metabolic pathways contribute to HCC progression and recurrence. Upregulation of galactokinase and downregulation of fructose-1,6-bisphosphate expression might result in the channeling of glucose and derivatives into the glycolytic pathway, which is the main source of energy production for tumor cells in the phenomenon termed the "Warburg effect" (Warburg 1956). The decrease in expression of enzymes involved in folate and amino acid metabolism may reflect a general reduction in liver function. In particular, downregulation of S-adenosylmethionine synthase, the enzyme essential for formation of S-adenosylmethionine (AdoMet) in the methylation cycle of the liver, is reported in two independent publications. Chronic decrease in AdoMet results in the development of HCC in mouse models (Martinez-Chantar et al. 2002), and proteomic studies by Liang et al. and Sun et al. demonstrated the downregulation of S-adenosylmethionine synthase in HBV-associated HCC tissues, suggesting the

Table 2 Proteins with consistent regulation patterns reported in multiple publications. This table shows the list of proteins that have similar regulation patterns reported in two or more publications. The expression levels of these proteins are not validated in the respective publications. The proteins are ordered according to their regulation patterns and key functions (↑ = upregulated in recurrence, ↓ = downregulated in recurrence)

UniProt accession number	Protein name	Key function	Regulation	References
P12004	Proliferating cell nuclear antigen	DNA replication and repair	↑	Orimo et al. (2008)
				Cheng et al. (2011)
P61978	Heterogeneous nuclear ribonucleoprotein K	Nucleic acid binding	↑	Bai et al. (2009)
				Taoka et al. (2014)
P02751	Fibronectin	Cell adhesion and cell motility	↑	Bai et al. (2009)
				Taoka et al. (2014)
P60709	Actin, cytoplasmic 1	Cytoskeletal protein	↑	Cheng et al. (2011)
				Kanamori et al. (2011)
P07437	Tubulin beta chain	Cytoskeletal protein	↑	Cheng et al. (2011)
				Kanamori et al. (2011)
P04792	Heat shock protein beta-1	Molecular chaperone	↑	Cheng et al. (2011)
				Taoka et al. (2014)
P51570	Galactokinase	Carbohydrate metabolism	↑	Tan et al. (2014)
				Taoka et al. (2014)
P09467	Fructose-1,6-bisphosphatase 1	Gluconeogenesis	↓	Orimo et al. (2008)
				Taoka et al. (2014)
O75891	Cytosolic 10-formyltetrahydrofolate dehydrogenase	Folate metabolism	↓	Bai et al. 2009
				Cheng et al. (2011)
O95954	Formimidoyltransferase-cyclodeaminase	Amino acid and folate metabolism	↓	Orimo et al. (2008)
				Cheng et al. (2011)

(*continued*)

Table 2 (continued)

UniProt accession number	Protein name	Key function	Regulation	References
Q00266	S-Adenosylmethionine synthase isoform type-1	Amino acid metabolism	↓	Orimo et al. (2008)
				Cheng et al. (2011)
P32754	4-Hydroxyphenylpyruvate dioxygenase	Amino acid metabolism	↓	Orimo et al. (2008)
				Cheng et al. (2011)
				Taoka et al. (2014)
O95154	Aflatoxin B1 aldehyde reductase member 3	Aldehyde metabolism, detoxification)	↓	Orimo et al. (2008)
				Taoka et al. (2014)
P30084	Enoyl-CoA hydratase, mitochondrial	Lipid metabolism	↓	Orimo et al. (2008)
				Tan et al. (2014)
P00441	Superoxide dismutase [Cu-Zn]	Antioxidant	↓	Yokoo et al. (2007)
				Cheng et al. (2011)
P30039	Phenazine biosynthesis-like domain-containing protein	Unknown	↓	Orimo et al. (2008)
				Cheng et al. (2011)

involvement of decreased AdoMet levels in HCC progression (Liang et al. 2005; Sun et al. 2007). Increased oxidative stress has also been correlated to an increased risk of HCC recurrence (Suzuki et al. 2013), which may be reflected by decreased levels of antioxidative enzymes such as superoxide dismutase. Overall, the list of proteins indicated the changes in expressions of these proteins that promote HCC progression are also involved in increased risk of recurrence, and thus they could be potential prognostic markers for HCC.

Future Perspectives

Despite the numerous publications that used omics-based methods in identification of predictive biomarkers for HCC recurrence, none of the suggested markers are currently used in the clinic. Furthermore, current treatment modalities are limited to early-stage cancers, with sorafenib being the only treatment available for late-stage

HCC patients. Currently, there are no medications available that show efficacy in preventing HCC recurrence. In the following sections, the improvements that can be made in future biomarker discovery and clinical translation will be discussed.

Selection of Patients for the Discovery Phase

Standardization of inclusion criteria for the samples used in the discovery phase would yield more meaningful results for clinical use. Improvements in imaging techniques have resulted in an increased numbers of HCC patients diagnosed in the early stages. The tissue samples analyzed in the discovery phase should preferably include early-stage patients with well-differentiated tumors and similar etiology to minimize confounding factors that might influence the data obtained from the proteomic studies. Subsequently, patient cohorts with mixed etiology and tumor stage could be used during the validation phase to ascertain the applicability of the protein signatures for recurrence prediction in the clinical setting.

Use of Clonal Analysis in Determination of Intrahepatic Recurrence

The cutoff time for early recurrence is not standardized and varies between 1 and 2 years in the literature. The use of a cutoff time may not be biologically relevant, as de novo synthesis of new tumors may occur within 2 years after resection. Furthermore, in patients exhibiting multifocal HCC during recurrence, it is difficult to determine the origins of the tumors. Hence, clonal analysis of both recurrent and primary tumors should be carried out to confirm the true "relapse" of HCC. These cases may then be selected and classified as the "true" intrahepatic metastasis/relapse, and the primary tumor proteome can be compared with other HCC cases with no relapse or with multicentric occurrence. This approach has recently been applied to two patients (Miao et al. 2014), which combined the use of whole genome sequencing, RNA sequencing, and single-nucleotide polymorphism analysis to determine tumor clonality and identify differentially expressed genes and pathways between tumors arising from intrahepatic recurrence and multicentric carcinogenesis. A similar approach combining clonal analysis and the use of proteomics is recommended for future studies to minimize confounding factors introduced by inclusion of patients suffering from multicentric carcinogenesis.

Identification of Molecular Pathways and Drug Targets in Proteomic Studies

Most of the current proteomic studies focused on identification of single biomarker that can best discriminate patients with or without early recurrence. However, cancer is a heterogeneous disease resulting from accumulation of genetic

aberrations and the interaction with environmental factors; hence, a single biomarker may be insufficient for prediction of recurrence. Furthermore, changes in tumor proteome may have synergistic or redundant effects that contribute to tumor progression and survival. Redundancies in molecular pathways contribute to the failure of molecular-based therapies for cancer treatment. Hence, it may be beneficial to look at molecular and signaling pathways that are dysregulated in tumors of recurrent cancers. A combination of transcriptomic and proteomic data may provide information with regard to the overexpression of specific transcription factors, which can be linked to the change in expression levels of downstream protein targets. This might allow us to identify suitable protein targets within the pathway such as receptor molecules and kinases that are more amenable for targeting by small molecules.

Identification and Validation of Potential Biomarkers in Serum/Plasma

Current efforts have been placed in identification of protein biomarkers using tissue samples. However, it is not feasible to perform repeated sampling of liver tissues for surveillance. Biofluids such as serum and plasma are good sources for proteomic analysis as they can be obtained from patients with minimal invasiveness. Most of the clinically approved biomarkers for cancer detection and monitoring are serum based (Fuzery et al. 2013). Hence, despite the challenges faced by serum and plasma proteomics (Anderson and Anderson 2002), it may be worthwhile to use these biofluids as samples for biomarker discovery. Alternatively, if the proteomic studies on tissues identified known secreted proteins, their expression levels in serum/plasma could differ. This could result in the development of a serum-based biomarker which could be translated into clinical practices.

Push for Better Commercial Antibodies for Validation of Candidate Biomarkers

Validation of proteomic data usually involves the use of antibody-based detection methods, such as immunohistochemistry and Western blotting, in determination of expression levels of protein of interest in a separate sample cohort. The availability and quality of antibodies are of upmost importance for obtaining reliable information. The Human Protein Atlas project was initiated to fulfill this unmet need by generating high-quality antibodies as well as validating existing commercial antibodies (Berglund et al. 2008; Ponten et al. 2008; Uhlen et al. 2015). The Human Protein Atlas portal is publically available (www.proteinatlas.org) and showcases spatial expression of proteins in different human tissues and cancer types, as well as cancer cell lines. This is a big step to improve the quality and number of antibodies available for validation purposes.

Alternative Validation Methods

Improvements in mass spectrometry have led to the possibility of reproducible quantitation of proteins across different types of biological samples and a wide dynamic range of protein detection. This can be achieved by targeted methods such as selected reaction monitoring (SRM), in which precursor/fragment ion pairs that uniquely represent a peptide are first selected and protein quantitation is obtained by comparison of signals obtained from the transition signals to a suitable reference. The analysis method could also be applied to data from SWATH-MS. Similar techniques are currently in use for routine monitoring of small molecules in clinical labs, which would aid the transition from the bench to the clinic.

Other Potential Applications of Proteomics in Cancer

In the review, we have highlighted the use of proteomics in identification of predictive markers for early HCC recurrence. Despite the progress in separation and mass spectrometry technologies, the number of clinically approved biomarkers arising from proteomic studies is dismal. OVA1 remains as the one and only clinically approved panel of five protein biomarkers for detection of ovarian cancer. These five proteins include apolipoprotein A1, prealbumin, transferrin, beta-2 microglobulin, and CA 125II, of which the first four proteins were identified by SELDI-TOF-MS (Zhang et al. 2004). We believe that the combination of a good study design, coupled with the rapid improvements in mass spectrometry technology, would result in a panel of biomarkers with high sensitivity and specificity for prognosis of HCC.

Proteomics can also be applied in understanding the mechanisms of existing cancer drugs. This would unravel the mechanisms of drug's action and drug resistance as well as identify predictive biomarkers for drug response. Such studies had been performed on genistein (Narasimhan et al. 2015), paclitaxel (Xu et al. 2015), and sorafenib (Yeh et al. 2015). This forms the basis of personalized medicine, where medications would be matched to individual cancer patients based on the characteristics of the tumor proteome and the mechanism of drugs' action.

Summary Points

- Recurrence is the main cause of death for patients who received curative treatment for hepatocellular carcinoma.
- Recurrence can be classified into two main forms, namely, intrahepatic metastasis and multicentric carcinogenesis.
- Clonal analysis indicated that the recurrent tumors from intrahepatic metastasis originate from the primary tumor, and these tumors are usually more aggressive and lead to poor prognosis.

- Most of the proteomic studies interrogate the proteome of the primary tumor to unravel biomarkers for prediction of early recurrence.
- Proteomic techniques used in the studies include gel-based, liquid chromatography-based, and chip-based platforms.
- Candidate biomarkers identified in these studies have diverse molecular functions. They include heat shock proteins, cytoskeletal proteins, and metabolic enzymes.
- Functional validations in cell lines implicate the overexpression of these proteins in promoting metastasis and cell proliferation.
- Improvements can be made in the study design for discovery of predictive biomarkers. This includes the careful selection of patients based on tumor staging and inclusion of clonal analysis of the primary and recurrent tumor/s.

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