Minireview

Proteomics of Hepatocellular Carcinoma in Chinese Patients

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Abstract

Hepatocellular carcinoma (HCC) is a malignant tumor of liver that causes approximately half a million deaths each year, of which over half of the cases are diagnosed in China. Because of its asymptomatic nature, HCC is usually diagnosed at late and advanced stages, for which there are no effective therapies. Thus, biomarkers for early detection and molecular targets for treating HCC are urgently needed. With the advent of high-throughput omics technologies, we have begun to mine the genomics and proteomics information of HCC, and most importantly, these data can be integrated with clinical annotations of the patients. Such new horizons of integrated profiling informatics have allowed us to search for and better identify clinically useful biomarkers and therapeutic targets for cancers including HCC. Capitalizing the large clinical samples cohort (over 100 pairs of tumor and matched adjacent nontumor tissues of HCC), we herein discuss the use of proteomics approach to identify biomarkers that are potentially useful for (1) discrimination of tumorous from nonmalignant tissues, (2) detection of small-sized and early stage of HCC, and (3) prediction of early disease relapse after hepatectomy.

The Unmet Medical Issues in Hepatocellular Carcinoma (HCC)

HCC IS A PREVALENT MALIGNANCY with poor outcome. About 600,000 new cases are diagnosed each year, among which 55% are identified in China, and increasing incidence is observed in Western countries. Globally, HCC remains the fifth most common cause of cancer mortality (Parkin et al., 2005). The major risk factors for HCC are cirrhosis related to chronic hepatitis B or C viral infection, environmental poisoning (e.g., alfatoxin), alcohol abuse, and genetic alteration (Pang et al., 2008). HCC usually progresses slowly and is asymptomatic in early stages. The most common curative treatments for HCC are surgical resection and liver transplantation; however, only 10–20% of the patients are eligible for the surgical intervention, and patients who had HCC resection often suffer from high rate of tumor recurrence (Poon et al., 1999). Other treatment options for patients with inoperable HCC are trans-arterial chemoembolization (TACE), systemic chemotherapies, and molecular targeted therapy (the current FDA-approved drug, Sorafenib). It is well known that most of the HCC patients are refractory to chemotherapies; thus, the response of these treatments is often not satisfactory. As such, the 5-year survival rate of HCC remains at approximately 10–20% over these years (Poon and Fan, 2004).

To improve the clinical outcomes, screening of HCC at high-risk subjects (hepatitis and cirrhosis groups) allows us to detect the cancer earlier for effective therapeutic intervention.

Today, the combination of serum alpha-fetoprotein (AFP) measurement and hepatic ultrasonography are standard practice for monitoring and/or screening of the malignancy. However, these methods have limited sensitivity and/or specificity. Therefore, we have made great efforts to identify panels of biomarkers for potential clinical applications on diagnosis and prognosis of HCC. Proteomics is one of the highly active research areas in biomarker discovery (Feng et al., 2006; Santamaria et al., 2007; Sun et al., 2007). Our team has employed a systemic approach for characterizing genetic and proteomic networks that underlie hepatocarcinogenesis (Fig. 1). We aim to: (1) identify biomarker for detection of HCC at early stage, when the tumors can be treated by curative surgery and/or local ablation therapies; (2) develop molecular tools that can be used to stratify high-risk subgroup of patients that may be benefited from targeted therapies such as Sorafenib; and (3) develop new drugs that can target aggressive and malignant tumors and show survival advantages. In this article, we focus on the application of proteomics platform for discovery of liver cancer biomarkers in Chinese patients, and provide a brief summary of the candidates for early detection of HCC and prediction of tumor recurrence (Table 1).

Approach for HCC biomarker discovery

Biomarkers are molecules that are strongly correlated with physiological states or pathogens, which may be nucleic acids, proteins, and metabolites. They can be used in early detection of
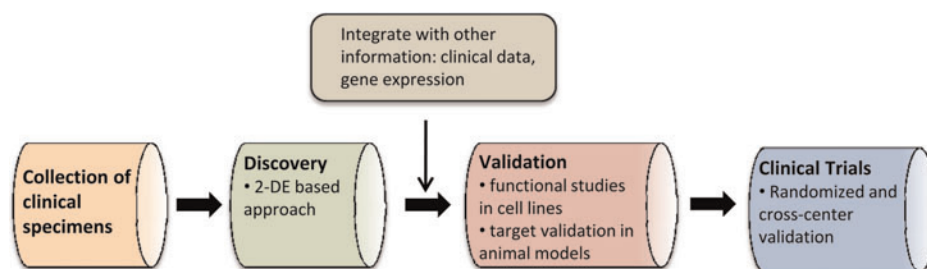
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FIG. 1. Systematic approach to HCC biomarkers and targets discovery. A systematic approach to HCC biomarkers and targets discovery involves four stages. (1) Clinical specimens such as tumor tissues, peripheral blood mononuclear cells, and serum samples are collected from HCC patients. (2) To understand how HCC can be differentiated at molecular levels, two-dimensional (2D) gel electrophoresis is used. Data are analyzed with combination of gene expression data. Also, associations between the molecular changes and clinical data of patients are analyzed. (3) A target's biological activities are being studied in cell lines and animal models for target validation. (4) Finally, the biomarkers or targets are tested in clinical trials before their uses in clinical settings.

a disease, personalized risk assessment, disease progression monitoring, and to differentiate responders from nonresponders to a treatment. The ideal markers should be highly expressed in the tumor tissues but not in the adjacent nontumor or normal tissues, and present in the patient's serum or plasma samples. Today, AFP is a conventional biomarker of HCC, but several studies reported that it has a low detection sensitivity ($\sim$50–60%) and its diagnostic accuracy is unsatisfactory (Sherman, 2001; Stefaniuk et al., 2010; Trevisani et al., 2001).

Hepatic ultrasonography is the standard diagnostic imaging method for HCC; however, it is highly operator-dependent and often subjected to high false-negative rate particularly when the tumor nodule is less than 2 cm in diameter.

To search for HCC biomarkers, we first collected over 200 clinical samples of tumor (TU) and adjacent nontumor (AN) tissues resected from HCC patients underwent surgery. Most importantly, each tissue sample also comes with corresponding serum/plasma samples before and after surgery

TABLE 1. PROTEOMICS OF HCC FOR TUMOR CLASSIFICATION, DETECTION, AND PREDICTION OF RECURRENCE

	Specimens	Platforms	Protein biomarkers	Study
Classification/ prognostification	Frozen tissues	2-DE MALDI-TOF-MS	Heat-shock protein 27, heat-shock protein 70, and glucose-regulated protein 78	Luk et al., 2006
	Frozen tissues	2-DEMALDI-TOF-MS	Haptoglobin, cytochrome b5, progesterone receptor membrane component 1, heat shock 27-kDa protein 1, lysosomal proteinase cathepsin B, and keratin I	Lee et al., 2009
	Serum samples	2-DEMALDI-TOF-MS/MS	Heat-shock protein 27	Feng et al., 2005
Blood-based biomarkers for detection	Frozen tissues	2-DEMALDI-TOF/TOF	Lamin B1	Sun et al., 2010
	Frozen tissues	2-DEMALDI-TOF/TOF	Vimentin	Sun et al., 2010
	Serum samples	SELDI-TOF-MS	Five proteomic peaks with m/z values at 3324, 3394, 4665, 4795, and 5152	Lei et al., 2010
	Serum samples	SELDI-TOF-MS	prothrombin induced by vitamin K absence-II	Zinkin et al., 2008
	Serum Samples	SELDI-TOF-MS	Six proteomic peaks with m/z values at 3444, 3890, 4067, 4435, 4470, and 7770	Kanmura et al., 2007
	Serum Samples	SELDI-TOF-MS	Fragmented form of vitronectin	Paradis et al., 2005
Tumor recurrence	Frozen tissues	2-DEMALDI-TOF/TOF	Mortalin	Yi et al., 2008
	Frozen tissues	2-DE and MS	APC-binding protein EB1	Orimo et al., 2008

2-DE, two-dimensional gel electrophoresis; MALDI-TOF-MS, matrix-assisted laser desorption/ionization time time-of-flight mass spectrometer; SELDI, surface-enhanced laser desorption/ionization.
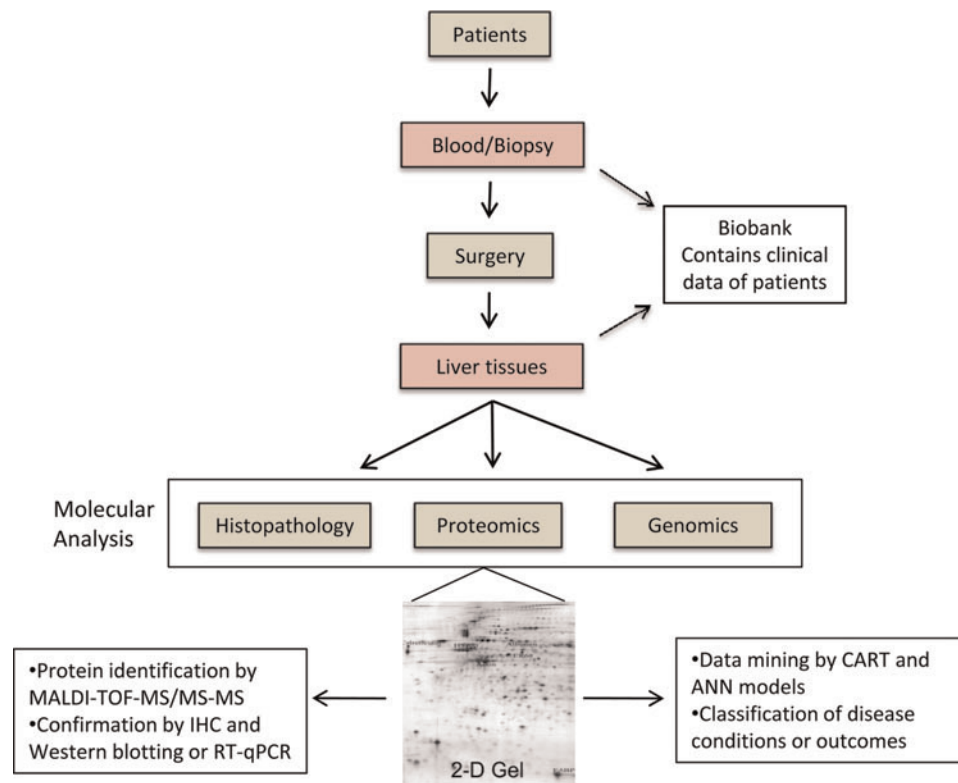
FIG. 2. Biobank as the interface between tissue donors and research scientists. Blood and liver tissue samples are collected from patients. Clinical data such as demographic information (e.g., gender, age, race), clinicopathological information (e.g., HBV or HCV infection, serum AFP level, tumor stage), and clinical outcomes (e.g., survival times) are collected from patients and maintained by biobank. Various technologies such cDNA microarray and two-dimensional (2D) gel electrophoresis are used to reveal genetic or proteomic changes underlying HCC. The candidate proteins are characterized by matrix assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) and confirmed by immunohistochemistry (IHC), Western blotting, or real-time quantitative PCR (RT-qPCR). On the other hand, the 2D gel data are analyzed by artificial neural network (ANN) and classification and regression tree (CART) models to determine their associations with prognostic features and outcomes of patients.

from the tissue repository or biobank (Fig. 2) as well as with detailed demographic and clinicopathological data (Hao et al., 2009). For proteomics analysis, fresh frozen tissues were subjected to two-dimensiontal gel electrophoreses (2-DE), and subsequently the proteomic profiling data were analyzed using different data-mining algorithms, such as artificial neural network (ANN) and classification and regression tree (CART) (Luk et al., 2007). Differentially expressed proteins were selected for MALDI-ToF/MS analyses. Protein identification was further confirmed by qPCR, Western blot and immunohistochemistry methods on separate set of clinical samples. Clinical correlation analysis of each candidate protein biomarker was performed to search for associations with prognostic outcomes and clinicopahtologic features of patients such as tumor size, differentiation, staging, vascular invasion, recurrence, and survival rate (Lee et al., 2007). Next, we developed immunoassays for measuring the serum biomarker levels in HCC patients and control cohorts (healthy volunteers, hepatitis and non-HCC cirrhosis patients).

Tissue proteome: biomarker set distinguishes HCC from nonmalignant phenotype

At first, we profiled 67 pairs of tumor and adjacent non-tumor HCC tissues as well as 12 normal liver tissues. The most striking finding is that certain chaperones or heat-shock proteins (HSPs) are frequently upregulated in the tumor samples, perhaps reflecting the stress conditions of cancer cells that need to overcome. We found Hsp27, Hsp70, and glucose-regulated protein (GRP)78 highly upregulated in HCC, which also have significant high receiver operating characteristic curves (ROC) values in distinguishing HCC from nonmalignant tissues. Their expressions profiles were confirmed by Western blotting and immunohistochemistry.

Aberrant expression of these HSPs was associated with clinic-pathological parameters, such as AFP level and tumor venous infiltration (Luk et al., 2006). In fact, other proteomic studies also revealed the deregulation of HSPs in HCC (Codarin et al., 2009; Shen et al., 2006; Zhang et al., 2007). Members of HSP family have been known to promote tumorigenesis. A recent study showed that the knockdown of Hsp27 significantly suppressed cell migration, invasion, and induced apoptosis in HCC cell lines (Guo et al., 2009). Moreover, a serum proteome analysis showed that Hsp27 was detected in 90% of HCC sera but not in normal sera (Feng et al., 2005). These findings suggest that HSPs have important role in human cancer, representing promising candidate biomarker for HCC.

Next, we expanded our cohort to 100 pairs of TU/AN HCC samples for 2-DE profiling analysis, and 80 pairs of the proteomic datasets passed the quality filter criteria (Lee et al.,

2009). We randomly divided the samples into training and validation subsets, and used the CART algorithm to analyze the data. The six protein markers identified in the study include haptoglobin, cytochrome b5, progesterone receptor membrane component 1, heat-shock 27-kDa protein 1, lysosomal proteinase cathepsin B, and keratin I. These proteins were used to develop a classifier model for predicting HCC. Using both the leave-one-out procedure and independent validation, we found the model has an overall sensitivity and specificity of 92.5%. Moreover, the protein levels of these biomarkers are significantly associated with serum AFP, total protein levels, and the Ishak's score.

Lamin B1 and vimentin are candidate biomarkers for early detection of HCC

Today, there are limited studies in revealing biomarkers that are specific for small-sized HCC. To identify biomarkers useful for early detection of HCC, we analyzed protein expression profiles between control subjects and HCC tumors of different stages (39 HCCs, 20 cirrhosis, and 16 nondiseased subjects). Twelve proteins were found significantly dysregulated in small-sized (≤ 2 cm) tumors—11 proteins (Vimentin; HSP90; GRP78; Cathepsin D; Lamin B1; Alternative splicing factor ASF-2; mitochondrial aldehyde dehydrogenase complexed with Nad+ and Mg2 Cys302ser mutant chain H; Keratin 10; Mitochondrial aldehyde dehydrogenase 2, precursor; Transferrin; Phosphoinositol 4-phosphate adaptor protein-2) upregulated and 1 protein (aldehyde dehydrogenase 4A1) downregulated. Based on the protein scores, sequence coverage, and expression ratios, we subsequently selected lamin B1 and vimentin for further study on its applicability to detect early stage and small tumor of HCC, respectively. Interestingly, lamin B1 mRNA was also found in patients' plasma as well. The expression of laminB1 was positively correlated with tumor stages, tumor sizes, and number of nodules (Sun et al., 2010b). Also, the use of circulating lamin B1 mRNA in plasma could detect early stages of HCC patients, with 76% sensitivity and 82% specificity.

Vimentin is the second candidate biomarker for early detection of HCC, which was significantly overexpressed in HCC, particularly in a subgroup of patients with tumor size less than 2 cm ($p < 0.01$). An indirect ELISA assay was developed using commercially available polyclonal antibody and in-house purified recombinant protein. We analyzed the serum levels of vimentin in a cohort of 88 HCC patients and 64 control subjects, and found that the vimentin level was significantly higher in small tumors than the nonneoplastic controls ($p < 0.01$). Moreover, the serum vimentin (at a cutoff level 245 ng/mL) had better sensitivity (40.91%) and specificity (87.55%) than AFP (cutoff level at 100 ng/mL; 30.23% sensitivity and 85.19% specificity) in detecting small-size tumors. Furthermore, combination of the two markers can enhance the detection rate of small HCC to 58.77% sensitivity and 98.15% specificity (Sun et al., 2010a). Nevertheless, serum vimentin level is being optimized to enhance the detection rate to 81%. In the longer term, development of monoclonal antibodies directed against vimentin isoform(s) that are specific in HCC would be necessary for more robust and specific biomarker assays. One study reported that the combined use of AFP, Lens culinaris agglutinin-reactive AFP (AFP-L3), and prothrombin induced by vitamin K absence-II (PIVKA-II) cor-

rectly identified HCC (≤ 2 cm) in 88% of the patients (Zinkin et al., 2008). Among the eight patients with tumor size less than 2 cm, seven were identified correctly using the signature.

Vimentin has been widely used as a mesenchymal marker for epithelial–mesenchymal transition (EMT), a process associated with malignant progression. Whether vimentin is solely a marker for EMT or has a function in promoting tumor progression remains to be elucidated. We hypothesize that vimentin (in the precursor form or splice isoforms to be determined) is secreted into the circulation during early stages of tumorigenesis, although further study is needed to reveal the mechanisms and biological functions.

Serum proteome: circulating markers or proteomic features for early detection of HCC

For screening purpose, a noninvasive blood test is of paramount importance for safety issues and cost effectiveness. Suspicious subjects can be further examined by needle biopsy and diagnostic imaging methods. Nevertheless, the current standard biomarkers are ineffective and inaccurate of diagnosing early staged HCC. Of particular, tumor size at 2 cm or less is extremely difficult to detect in serum samples by current biomarker assays or by ex vivo imaging in the tissues. Indeed, patients with small tumor in early stages can be cured effectively by surgery and local ablation therapies with good clinical outcomes.

Surface-enhanced desorption/ionization time-of-flight mass spectrometry (SELDI-TOF-MS) and ProteinChip technology require small amount of input material and enable quantitative measurement of specific protein in a high throughput manner. These features make them an efficient and sensitive platform for discovering biomarkers in serum. Using these technologies, we analyzed serum samples from a large cohort including 120 HCC patients and 120 chronic hepatitis-related liver cirrhosis patients. In this study, most of the HCC samples were obtained from early-staged HCC patients. We found that the serum SELDI-TOF proteomic signature, which is based on five proteomic features, in combination with AFP marker enhanced the detection of HCC to sensitivity of 95% and specificity of 98%, which promises to be a good tool for screening of HCC in at-risk population (Chen et al., 2010). Another study also demonstrated the capability of using SELDI-TOF-MS in analyzing serum samples from HBV-related HCC (He et al., 2008). On the other hand, Paradis et al. (2005) found a fragmented form of vitronectin as a novel serum marker in HCC, whereas Kanmura et al. (2007) demonstrated that the protein markers identified using SELDI-TOF-MS could diagnose six of the seven patients who had HCC. Although there is a great concern of the experimental variability about the SELDI assay, we believe that such issues can be resolved if the samples preparation and assay performance are done according to well-optimized standard operating procedures in a certified reference diagnostic laboratories.

Predictive biomarker for HCC recurrence

Tumor recurrence is the main cause of long-term death of patients with HCC resection, and it is clinically relevant to identify biomarkers that can predict early tumor recurrence after surgery or other curative treatments. We used differential proteomic approach to profile tumorous and matched

noncancerous tissues from 103 HCC patients, and found mortalin (HSP9A) was significantly upregulated in the "early-recurrence" subgroup of HCC patients. These patients with high mortalin expression levels had high rate of recurrence within 1 year after curative surgery. In addition, the mortalin expression level was associated with vascular invasion and short disease-free survival time (Yi et al., 2008). Interestingly, metastatic HCC cell lines showed higher level of mortalin mRNA compared to the nonmetastatic counterparts or primary HCC cell lines. It is suggested that mortalin is related to metastatic property of HCC having a potential clinical utility for prediction of disease relapse.

Other study employing proteomic profiling of 27 tumor and 11 adjacent nontumor tissues revealed APC-binding protein EB1 as an independent prognostic factor for recurrence (Orimo et al., 2008). Another report analyzed proteomic profiles of 12 patients with intrahepatic recurrence within 6 months postsurgery with those of 15 patients who had no recurrence within 2 years, and the group identified 23 protein spots that were associated with early intrahepatic recurrence (Yokoo et al., 2007). These proteins were involved in wide range of biological processes. It should be noted that patients recruited in the study are mainly HCV positive, whereas our HCC cohort is HBV related. Thus, it would be necessary to determine if those biomarkers are etiology related, and can be applicable to different ethnic groups.

Conclusion

Proteomic profiling is a powerful tool for discovering novel biomarkers and identification of new therapeutic targets. With the advent of the latest proteomic methods such as Multiple Reaction monitoring (MRM) assay in combination with Stable Isotope Standards with Capture by Anti-Peptide Antibodies (SISCAPA), high throughput detection of low-abundance proteins becomes tangible and feasible. There is a plethora of biomarkers reported for detecting liver cancer; however, very few, or none, of them have been validated in clinical studies. Multicenter validation on a select panel of biomarkers (in combination with AFP) would be necessary prior to clinical practice. We believe biomarkers for HCC detection and risk assessment will be on track to be tested in prospective, multicenter clinical trial studies.

Author Disclosure Statement

The authors declare no competing financial interest in this study.

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