**Toward the discovery of new biomarkers of hepatocellular carcinoma by proteomics**

Enrique Santamaria, Javier Munoz, Joaquin Fernandez-Irigoyen, Jesus Prieto and Fernando J. Corrales

Division of Hepatology and Gene Therapy, Laboratory of Proteomics, CIMA, University of Navarra, Pamplona, Spain

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**Correspondence**

Fernando J. Corrales, Division of Hepatology and Gene Therapy, CIMA, Facultad de Medicina, University of Navarra, 31008 Pamplona, Spain.
Tel: +34 948 194700
Fax: +34 948 194718
e-mail address: fjcorrales@unav.es

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**Abstract**

Primary liver cancer is the fifth most frequent neoplasm and the third most common cause of cancer-related death, with more than 500,000 new cases diagnosed yearly. The outcome for hepatocellular carcinoma (HCC) patients still remains dismal, partly because of our limited knowledge of its molecular pathogenesis and the difficulty in detecting the disease at its early stages. Therefore, studies aimed at the definition of the mechanisms associated with HCC progression and the identification of new biomarkers leading to early diagnosis and more effective therapeutic interventions are urgently needed. Proteomics is a rapidly expanding discipline that is expected to change the way in which diseases will be diagnosed, treated, and monitored in the near future. In the last few years, HCC has been extensively investigated using different proteomic approaches on HCC cell lines, animal models, and human tumor tissues. In this review, state-of-the-art technology on proteomics is overviewed, and recent advances in liver cancer proteomics and their clinical projections are discussed.

Primary liver cancer is the fifth most frequent neoplasm and the third most common cause of cancer-related death, with more than 500,000 new cases diagnosed yearly (1). Hepatocellular carcinoma (HCC) is a major health problem in Asia and Africa with the incidence steadily increasing in western countries (1). The major causes of HCC are currently known, including infection with hepatitis B (HBV) and C (HCV) viruses, ingestion of aflatoxin-contaminated food, and alcohol abuse (2). Other less prevalent risk factors have also been described such as iron or copper deposition or non-alcoholic steatohepatitis (NASH) (3). Hepatocarcinogenesis is a slow multistep and multifactorial process, usually a consequence of long-term inflammation and fibrosis, that involves the progressive accumulation of changes at the level of gene and protein expression (4). At the early stages, these alterations modify the phenotype of hepatocytes, resulting in the generation of cellular intermediates that finally evolve to HCC. Although the main risk factors are known and the population at risk are routinely screened, the prognosis of these patients is poor (5) mainly owing to the aggressiveness of the lesions at the time of diagnosis and to the lack of effective therapies. At present, only five biomarkers for HCC are available for clinical use (6), from which only α fetoprotein partially fulfils the requirements of an ideal tumor marker (6). Therefore, the application of new technologies to extend our knowledge about the molecular pathogenesis of HCC, to identify biomarkers leading to an early diagnosis, and to define new therapeutic targets is of great interest.

The biology of HCC has been studied extensively. Changes in DNA methylation, HBV- or HCV-induced alterations, together with mutations and loss of heterozygosity in tumor suppressor genes or oncogenes, arise as trigger factors in the progression of preneoplastic lesions (4, 7). However, different genomic studies devoted to defining the molecular pathogenesis of HCC resulted in dissimilar profiles of up- and downregulated genes (8–10). These differences might have resulted from the distinct etiology and differentiation state of the analyzed tumors and suggest that diverse, and perhaps, redundant mechanisms are involved in the control of cell proliferation protecting cells from neoplastic transformation.

Proteins are the cellular components performing and regulating most biological functions and, therefore, the systematic analysis of the whole proteinic complement of cells is needed to provide a functional meaning to the information provided by genome-wide gene expression studies. The moderate correlation...
between the expression of a gene and its functional product (11, 12), the added complexity resulting from protein posttranslational modifications, interactions, and subcellular location that confer or modify protein activities, and the limited presence of mRNA in biofluids, precluding the identification of relevant disease hallmarks, highlight the urgent need of studies at the protein level. These studies will surely promote the identification of novel biomarkers and a better understanding of the pathophysiology of disease contributing to the development of new strategies for the diagnosis and treatment of human disease. Proteomics is an emerging discipline that provides the means to perform systematic analysis of whole proteomes by a combination of different strategies to resolve complex mixtures of proteins and peptides from biological systems with mass spectrometry (MS) approaches aimed at the identification and characterization of the proteins of interest. Although comprehensive analysis of whole proteomes is still a challenging task, in the last few years, an increasing number of panels of proteins have been proposed as markers with central roles in the progression of human diseases based on differential proteomic studies. This review summarizes briefly state-of-the-art technology on proteomics, as well as recent advances in proteomics research on HCC. The impact of these studies on the clinical side is also discussed.

Proteomics, general considerations and technical approaches

The term proteomics was coined about 10 years ago, although the systematic analysis of proteins integrating biological systems appeared in modern biology in the early 1980s (13). Since then, proteome research has undergone tremendous advances on the technical side that provide the means to examine proteomes with an increasing efficiency. The application of these new advances to the understanding of the molecular mechanisms involved in cellular function and human disease has created a great expectation in the biomedical and pharmaceutical field. However, proteomic analysis is still an extremely challenging task a rising from the inherent complexity of the proteome. Separation, identification, and characterization of hundreds of thousands of different protein species present in the cell or biological fluids in a wide dynamic range of concentrations cannot be fully accomplished by a single experimental approach. Proteomics defines a broad range of activities, methods that provide complementary information about the proteome under study, and goals. Therefore, it is essential to define carefully the purposes of each study to delineate an appropriate workflow integrating the ideal combination of analytical procedures to generate consistent answers. Proteomic methods and technologies have been extensively reviewed (14, 15); therefore, we only summarize here the most relevant aspects of this expanding field.

In general, independent of the aim of the analysis, a proteomic investigation integrates four stages (Fig. 1): sample collection, storage, and protein solubilization, protein or peptide separation, protein identification, and integration of the information by bioinformatics. Normalized strategies for sample collection and storage are essential to ensure proteome integrity and analytical reproducibility. This is especially relevant in the case of human samples that, additionally, should be accompanied by precise diagnostics to permit correlations between clinical phenotypes, histologic alterations, and molecular parameters. Proteins must then be extracted and solubilized in buffers containing high concentrations of chaotropic agents, reducing agents, and detergents. The protocols must be optimized for each type of biological sample to improve the extraction efficiency but minimizing the interaction with the subsequent analytical procedures (16). Proteome coverage is greatly enhanced by the use of fractionation strategies and the subsequent study of subproteomes. Conventional methods generally based on differential solubilization or centrifugation of sucrose gradient to isolate subcellular organelles, as well as different commercial kits, are available to obtain fractions enriched on proteins sharing biochemical properties, functions, or subcellular location. Although these approaches obviously increase the complexity of the analysis, the limitation imposed by the wide range of protein abundance and the protein diversity on the detection of less abundant proteins might be partially overcome by the analysis of different subcellular fractions.

Protein separation is usually based on multidimensional electrophoretic and/or chromatographic procedures. Two-dimensional polyacrylamide gel electrophoresis (2-DE) is the most widely used method to resolve complex mixtures of proteins according to two biochemical parameters: the isoelectric point (pI) in the first dimension and the molecular weight (Mr) in the second dimension. This is a relatively simple method that allows visualization of thousands of proteins, detection of posttranslationally modified species, and targeting of protein expression alterations. However, different restraints are inherent to 2-DE such as relatively low sensitivity and throughput, reproducibility of gels, and limited resolution of membrane or...
extreme pI and Mr proteins. Some of these constraints can be circumvented by using the differential in-gel electrophoresis (DIGE) (17). In DIGE, proteins from two different samples to be compared are labelled using different fluorescence dyes (Cy2, Cy3, or Cy5), mixed equally, and resolved by 2-DE. Scanning at different wavelengths provides multiple images corresponding to different samples and therefore protein levels can be compared within a single gel by reducing gel-to-gel variability and improving accuracy in protein semiquantitation (18, 19).

As an alternative to 2-DE, shotgun proteomics has evolved. Similar to shotgun sequencing of genomic DNA, shotgun proteomics involve the proteolytic digestion of the protein mixture before fractionation. The generated peptides are then separated in one or more consecutive steps of liquid chromatography, analyzed by tandem MS (MS/MS), and the information is processed with bioinformatic tools to obtain the composition of the original sample. This approach can be used to compare protein profiles between cell states using stable isotope labelling. Peptide mixtures from different samples can be chemically (isotope-coded affinity tag, ICAT; isobaric multiplexing tagging system, ITRAQ), enzymatically (digestion in H\textsubscript{2}O\textsubscript{2}, or metabolically (stable isotopic labelling with amino acids in cell culture, SILAC; \textsuperscript{15}N) labelled using light and heavy isotopes, and then combined equally and analyzed by liquid chromatography electrospray tandem MS (LC ESI-MS/MS). The relative abundance of peptides is calculated by computer algorithms that calculate the ratio (light vs heavy) for each peptide pair. Differential protein expression profiling, protein interactions, or posttranslational modification identification can be alternatively accomplished by using protein chips (20, 21). Although protein arrays have tremendous potential, the development of protein microarrays for high-throughput proteomics investigation is slow because of the complex nature of proteins. In addition to macro- and microarrays, or microfluidic and microwell chips, alternative formats such as surface-enhanced laser desorption/ionization (SELDI) and surface plasmon resonance are already available. SELDI, in combination with time-of-flight MS (TOF-MS), is the most widely used protein chip format. SELDI TOF combines protein retention on chemically treated supports with specific chromatographic features (protein chip arrays) with TOF-MS analysis to generate specific signature patterns that can be compared to distinguish different samples (22).

Nowadays, protein identification is mainly achieved by MS. MS is a sensitive and reliable method to identify a protein from a single 2-DE gel spot, but also provides the capacity of high-throughput protein identification. MS analysis is performed on a mixture of peptide fragments generated from the proteolytic or

**Fig. 1.** Schematic representation of a typical experiment oriented to the identification of biomarkers of human disease based on proteomics approaches.
chemical cleavage of proteins. Peptides are then ionized using matrix-assisted laser desorption/ionization (MALDI) (23) or ESI (24) and their mass to charge ratio \((m/z)\) is measured in different types of instruments. The \(m/z\) ratios of peptide fragments configure the peptide mass fingerprint (PMF) of a protein and allow its identification by comparison with the theoretical PMFs of genome-wide protein sequence databanks. Protein identification can be further supported by MS/MS experiments that provide information relative to the amino acid sequence of peptide fragments. In tandem MS, a particular peptide/ion is isolated and fragmented by collisioning with an inert gas. A mass spectrum of the resulting fragments is then generated and interpreted. This information is compared with the theoretically predicted fragments for all possible peptide sequences in a database to identify the peptide sequence and, therefore, the original protein. MS instrumentation is evolving at a great speed, providing new generations of analyzers that ensure increasing sensitivity and mass accuracy and the development of new applications such as MS-based image analysis that allow protein profiling on cell or tissue specimens.

Data obtained from high-throughput proteomic analysis must be integrated and processed using appropriate bioinformatic tools to generate valuable biological information from the resulting complex molecular descriptions in formats allowing the validation of data and exchange among different laboratories.

**Differential proteomics of HCC: discovery of potential biomarkers**

The outcome for HCC patients still remains dismal, partly because of our limited knowledge of the molecular pathogenesis and the difficulty in detecting the disease at its early stages. Therefore, studies aiming at the definition of the mechanisms associated with HCC progression and the identification of new biomarkers leading to early diagnosis and more effective therapeutic interventions are urgently needed. In the last few years HCC has been extensively investigated using different proteomic approaches on HCC cell lines, animal models, and human tumor tissues.

HCC cell lines have been used as *in vitro* models for proteomic studies of HCC as they are relatively homogeneous systems when compared with liver cancer tissue, which is composed of multiple cell subpopulations. Additionally, cell lines allow functional studies to determine the effects of modulating the expression or activity of a particular protein. Based on the identification of differential proteins in HCC cells, these investigations have provided valuable information to recognize changes in cellular pathways that might participate in the development and maintenance of the transformed phenotype. Alterations of different metabolic enzymes, proteins involved in calcium homeostasis, cytoskeleton, or oxidative stress, reduced expression of tumor suppression factors or the upregulation of proteins increasing cell resistance to apoptosis such as heat shock protein 27 (HSP 27) have been identified in HCC cell lines (25–27). The re-expression of embryonic enzymes including \(\alpha\)-feto-protein, glutathione S transferase (GST) \(\pi\), hexokinase B, or aldolase A in HCC has also been reported (28).

The application of subfractionation strategies has extended the capacity to detect HCC differential proteins. Clifton et al. (29) showed that the selective solubilization of plasma membranes and subsequent comparison and identification of the proteins from normal liver and Morris hepatoma 7777 allow the recognition of members of HSPs and low Mr annexin families as markers of HCC. The mechanisms by which HBV and HCV promote HCC development have also been evaluated at the proteomic level using *in vitro* models. Using 2-DE analysis, it was found that the levels of a number of cellular proteins change after expression of hepatitis virus B HBx protein in non-HCC Chang cells (30). Cellular proteins interacting with the HCV core protein have been identified including citokeratins, vimentin, HSP 90, coflin, and low Mr phosphotyrosine protein phosphatase (31). Interactions of NS4A and NS5B HCV proteins with HSP 27 and an RNA-binding protein have also been reported (32). These findings might provide mechanisms by which HCV interferes with cellular pathways such as translation, replication, apoptosis, and cell signalling. Mannova et al. (33) reported the upregulation of proteins involved in cell signalling, protein transport, and vesicle formation in lipid raft fractions from HCV-replicating Huh7 cells. Additionally, among other alterations identified after comparison of HCC cell lines with different capacities of metastasis, it has been proposed that the upregulation of the calcium-binding protein S100A4 might be associated with the metastatic potential of tumor cells through the regulation of matrix metalloproteinase secretion and strengthened motility and invasion properties (34–36).

The identification of new diagnostic biomarkers and therapeutic targets for HCC is increasingly being approached by comparing protein profiles of diseased and non-diseased human samples, mainly serum and liver tissue. In the last case, the usual protocol consisted of paired analysis comparing tumors and
non-tumoral surrounding tissue from the same patient. It is important to note that this approach might conceal alterations common to peritumoral tissue and tumors, hampering the detection of proteins whose levels are already modified in hepatitis or cirrhosis, conditions at risk of HCC development (10). The most extended experimental approach is based on proteomic differential display on 2D gels in combination with different MS approaches. However, alternative methods have been also used including laser capture microdissection in combination with ICAT and 2D LC. This approach greatly enhanced the specificity at the level of cell type and resolved some of the limitations inherent to protein separation by 2-DE (37).

The biological and pathogenic activities of HCV and HBV are different, and, therefore, it is suspected that the process involved in the development of hepatitis and HCC might be distinct for the two viruses (38, 39). According to this idea, Kim et al. (40) showed that although protein alterations common to HCV- and HBV-related HCC can be identified, SOD specifically decreased in HBV-HCC patients, GABA-AT is upregulated only in HCV-HCC, and HSP 27 and enoyl-CoA hydratase are reduced in HBV and increased in HCV-related HCC. Overexpression of proliferating cell nuclear antigen and stathmin 1 have been proposed as biomarkers of HBV-associated HCC (6). Among other alterations, it has been shown that hepatic aldolase B is replaced by non-hepatic isoform A in HCV-related HCC, and proteolytic fragments as well as unexpected species of albumin and smoothelin, likely resulting from posttranslational modifications, were also found in diseased tissue (41). Although functional studies to link these alterations with hepatocarcinogenesis are mandatory, upregulation of APOE, CLIC 1 (chloride intracellular channel 1), and heterogeneous nuclear ribonucleoproteins in HCV-related HCC was reported (42). The expression of α-enolase has been positively correlated with tumor size and venous invasion in HCV-related HCC (43). Additionally, an increasing number of proteins arising from proteomic studies are proposed to be involved in the development of HCC, regardless of its etiology, which might extend our knowledge about the specific properties of the transformed cells. Overexpression of nuclear factor κB-associated factor Wnt-1 has been proposed as a mechanism of hepatocarcinogenesis common to HCV and HBV patients (44). The analysis of HCV, HBV, and cirrhosis-related HCC revealed upregulation of PDI A3 common to other cancers and hypoxia, as well as downregulation of SMP30, different metabolic enzymes involved in lipid catabolism, HSP 27 (in contrast to other studies showing upregulation of this protein), and cathepsin D, among others (45). Upregulation of three species of glutamine synthase (GS), likely corresponding to differentially phosphorylated states, has been reported in HCC tissue. This accumulation of GS isoforms might result from an increased expression through the Wnt-β-catenin pathway or from a decreased degradation by the proteasome (46). Studies comparing normal, cirrhotic, and HCC tissue revealed alterations common to cirrhotic and tumor samples and others that are specific to HCC such as downregulation of cytochrome B5, catalase, liver carboxylesterase, ADH, peroxiredoxin 3, sarcosine DH, or upregulation of HSP 70RY and nucleophosmin (47). Downregulation of different metabolic enzymes and cathepsin A, as well as upregulation of HSPs 27 and 70, GST Ω, and MAT, likely the non-hepatic isoform MATII, has been reported. Additionally, 14-3-3g protein is increased and this alteration might determine whether the cell undergoes proliferation or apoptosis (48). Luk et al. (49) found upregulation of HSPs 27, 70, and GRP78 in HCC. While no association of increased HSP 70 with any pathological feature was found, upregulation of HSP 27 has been linked to α-fetoprotein, and GRP78 enrichment was associated with tumor infiltration. These alterations are not strictly related to HCV or HBV infection. Proteins that have already been associated with HCC such as fatty-acid binding protein, HSP 27, S100, or cytokeratin 18 have also been related to mechanisms associated with the metastatic capacity of primary liver tumors (50). It has recently been reported that the thioredoxin-like protein Hcc-2 is overexpressed in poorly differentiated tumors but remains unchanged in well-differentiated HCC. Consequently, it might be used to assess the differentiation state of liver tumors (51). Upregulation of aldose-reductase-like protein (ARLP) arises as a specific marker of liver tumors. In tissue sections, it was observed that while the tumor cells are positive for ARLP, the surrounding cirrhotic or non-cirrhotic surrounding tissue was negative. Overexpression of this protein might improve the detoxification capacity of tumor cells and increase their resistance to chemotherapy (52).

The identification of biomarkers of HCC in biological fluids is especially attractive. Comparative inspection of normal and diseased serum proteomes is of particular interest as serum constantly perfuses tissues and, therefore, the onset or the presence of a disease may be determined by measuring and characterizing the thousands of individual circulating proteins and peptides. Changes at the proteome level in serum have also been used to monitor the effect of treatments...
applied to HCC patients (53). Haptoglobin, α1 antitrypsin, transthyretin, ApoAIV, isoforms of ApoAI, and a proteolytic fragment of topoisomerase IIB have been proposed as markers of HBV-associated HCC (54). A C-terminal fragment of complement C3 protein and an isoform of ApoA1 have been proposed as serum markers of HCC (55). HSP 27 has been identified in the serum of HCC patients although, as its presence has also been associated with other diseased conditions (56), its specificity requires further elucidation (57). The metastatic potential, aggressiveness, and poor differentiation state of liver tumors may be evaluated by the presence of the cytokeratin 19 fragment CYFRA21-1 in serum (58). Chignard et al. (59) have reported the identification of proteolytic fragments of RE proteins including calreticulin, PDIA3, PDI, and GRP78 in tumors, and those from calreticulin and PDIA3 were also detected in the serum of HCC patients. The levels of these two fragments in the serum of HCC and at-risk patients were significantly different. The analysis of serum glycoproteins has also provided potential biomarkers in the serum of patients with HBV-associated HCC such as serum amyloid component P, a proteolytic fragment of ceruloplasmin (60), and increased levels of fucosylation in serum immunoglobulins (61). It has also been reported that a unique pattern of Hp glycoforms with altered sialylation and fucosylation is specific of HCC and is associated with tumor progression (62). The application of SELDI TOF technology has provided HCC-specific protein profiles that can be used in diagnostics in combination or not with other markers. Specific profiles have been proposed based on clusters of differential peaks that allow identification of HCC among cirrhosis, hepatitis, and less severe liver alterations (63–65). From SELDI data, Paradis et al. propose 8.9 kDa as the highest discriminating peak, which was further identified as a C-terminal fragment of V10 vitronectin likely resulting from the activity of metalloproteases. SELDI TOF-based studies have also identified the complement C3a protein in serum from HCV-related HCC patients (66). There is increasing evidence for an immune response to cancer in humans, as indicated by the identification of autoantibodies to tumor antigens. Taking advantage of this biological response, different studies have aimed at the identification of autoantibodies in the serum of patients as markers of HCC. In these studies, samples are tested with autologous serum and the immunoreactive proteins are further characterized. Autoantibodies against calreticulin, cytokeratin 8, nucleoside diphosphate kinase A, and ATP synthase ßchain have been identified independently of the HBV or HCV status (67). More recently, autoantibodies against HSP 70, GA3PDH, peroxiredoxin, and Mn SOD have been described in the serum of NA/NB HCC patients (68).

The hundreds of differential proteins provided by studies on hepatoma cell lines and human samples have undoubtedly extended our knowledge about HCC at the molecular level. However, although some of these proteins can easily be associated with HCC, the protein repertoires reported are barely coincident and hence, the enormous variability among studies hampered the immediate identification of ready-to-use biomarkers (Fig. 2). This variability is likely associated with specific biological properties of the samples analyzed (different cell lines or HCC samples

Fig. 2. Protein profile of hepatocellular carcinoma (HCC). Differential proteins reported in 29 HCC studies including cell lines or tissue samples are represented. Up- and downregulated proteins are in red and green, respectively. Little coincidence was found among experiments, likely resulting from the biological heterogeneity of samples and from the different analytical procedures used. Some proteins commonly altered are indicated.
from different patients with heterogeneous genetic background, life style, etc.), the use of different reference samples, and the heterogeneity of the analytical procedures used. Additionally, HCC is a multifactorial and progressive disease that must be detected at the early stages to improve its clinical management effectively. The use of experimental models of hepatocarcinogenesis in which the genetic background and the environmental conditions have little impact on proteome variability, and that also facilitate the design of longitudinal studies from preneoplastic stages, may have a decisive impact in the identification of HCC-associated proteins (4). It has recently been shown that the ubiquitin-proteasome and lysosomal pathways are enhanced in HBx transgenic mice that develop HCC of longitudinal studies from preneoplastic stages, may have a decisive impact in the identification of HCC-associated proteins (4). It has recently been shown that the ubiquitin-proteasome and lysosomal pathways are enhanced in HBx transgenic mice that develop HCC by 18 months (69). The combination of data obtained from the analysis of a knockout (KO) mouse model of HBx transgenic mice that develop HCC by 18 months (69). The combination of data obtained from the analysis of a knockout (KO) mouse model (MAT1A−/−) with a chronic deficiency on hepatic S'-adenosylmethionine (AdoMet) with studies on human samples led to the identification of biomarkers and functional alterations associated with HCC. AdoMet has a critical role in maintaining normal hepatic function and tumorigenesis in the liver. AdoMet has generally been considered as a central intermediary metabolite involved in the synthesis of homocysteine and polyamines as well as the main cellular methyl group donor (70). However, recent evidences indicate that in addition to its central metabolic function, AdoMet is an intracellular control switch that regulates essential hepatocyte's functions such as proliferation, differentiation, and death (71). According to this, a deficiency of hepatic AdoMet synthesis has been associated with the progression of liver diseases (72–75). In the adult liver, AdoMet is the product of the enzyme methionine adenosyltransferase I/III (MATI/III) (76), which is encoded by the gene MAT1A (77). Early evidences showing that MATI/III activity is impaired in patients with hepatitis and liver cirrhosis (73, 74) and that AdoMet treatment increases the survival of patients with alcoholic liver cirrhosis (78) pointed out the crucial role of AdoMet in the maintenance of liver homeostasis. Moreover, targeted disruption of murine MAT1A, resulting in a chronic reduction of hepatic AdoMet, induces the spontaneous development of NASH and HCC at the age of 8 and 18 months, respectively, in the KO mice (79, 80). Proteomic analysis of KO livers revealed that the alteration of a network of mitochondrial proteins regulated by AdoMet, and the generation of oxidative stress long before any histological abnormality was detected are primary alterations that explain, at least partially, the molecular pathogenesis of this multistep disease (81). In agreement with the redox imbalance of the KO liver, a highly oxidized form of ApoA1 was found to be increased in the serum of MAT1A−/− mice months before the detection of liver tumors, as well as in patients with HBV-associated HCC (82). Proteomic analysis allowed the identification of 151 differential proteins in MAT1A−/− mice tumors. According to the notion that the tumor may progress through different pathways, proteomic profiles of different MAT1A−/− tumors showed higher variability values than those found in controls or other preneoplastic pathologica conditions associated with this model. Based on the comparison of tumors from the same and distinct livers, it was suggested that the stochastic accumulation of errors in the biological program of cellular systems during the development of tumors may explain only partially the increase of the proteomic variability. In addition, proteomic heterogeneity might also be contributed by individual-specific factors that may impose a selective pressure on preneoplastic foci conditioning their evolution according to a Darwinian selection system. Among all differential proteins, 27 changed in at least 50% of the analyzed tumors, and some of these alterations were already detected months before the development of HCC in the KO liver. In addition to the alteration of proteins involved in the AdoMet metabolism, also reported by Liang et al. (83), the expression level of genes coding for 13 of these proteins was markedly decreased in human HCC. Interestingly, seven of these genes were also found to be downregulated in a pretumoral condition such as cirrhosis, while the depletion of only one marker was assessed in less severe liver disorders (84).

Final considerations and perspectives

Proteomics is a rapidly expanding discipline with a tremendous potential to extend our understanding of essential aspects defining biological functions in living organisms, as well as providing information to better define the molecular pathogenesis of human diseases and to identify biomarkers improving patient diagnosis, treatment, and outcome. Although there are still some limitations that must be overcome before clinical applications based on new proteomic discoveries become a reality, this new discipline will provide in the near future predictive and individualized approaches to patient care promoting the development of selective treatment modalities to benefit the individual patient. New analytical strategies combining more efficient protein preparation and separation methods with high-performance MS approaches are expected to increase our capability to detect target proteins with
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clinal impact. The availability of MS instruments allowing the identification of posttranslational modifications such as phosphorylation (85) and glycosylation (86) is already promoting the discovery of essential information to understand the progression of complex diseases such as HCC. Protein profiling of biofluids using SELDI TOF represents one of the most promising methods for diagnosing and monitoring cancer by combining multiparametric biomarker analysis with artificial neural networks and pattern recognition (87). MALDI-MS tissue profiling improves cellular specificity, analytical throughput, and allows a combination of histological and proteomic assessment of sample regions (88, 89). In addition, the increasing availability of validated and well-characterized affinity capture reagents, such as antibodies or aptamers, is promoting the development of proteomics platforms for the prevention, early detection, and monitoring of cancer (90, 91). Finally, biomarker reliability is greatly benefited by the definition of common standards for data representation, and analysis, and the creation of data repositories to compare, exchange, and share data within the community (92). The methodological breakthrough that has taken place within proteomics in the last decade promises to have a major impact on clinical practice by promoting new ways in which diseases will be diagnosed, treated, and monitored.

As the liver performs multiple functions, no single laboratory test or battery of tests is sufficient to provide a complete estimate of hepatic function in every clinical situation. Moreover, the definition of complex disorders involving multiple underlying pathogenic mechanisms may require the recognition of a selective repertoire of molecular targets rather than individual markers. The pioneer studies revised herein highlight the enormous potential of proteomics in the investigation of HCC. It is expected that the clinical management of this disease will be benefited in the near future by the discovery of new biomarkers resulting from differential protein/peptide profiling. However, the complexity of HCC is still challenging for this still young science. Hepatocarcinogenesis is a slow and multifactorial process that involves the progressive accumulation of changes at the level of gene and protein expression (4). At the early stages, these alterations modify the phenotype of hepatocytes resulting in the generation of cellular intermediates that finally evolve to HCC. Different studies devoted to define the molecular pathogenesis of HCC resulted in dissimilar profiles of up- and downregulated proteins. The apparent discrepancy might result from the distinct etiology and differentiation state of the analyzed tumors and suggest that diverse, and perhaps redundant, mechanisms are involved in the control of cell proliferation protecting cells from neoplastic transformation. This is compatible with the notion that the tumor may progress through different pathways resulting in the molecular heterogeneity denoted by proteomic studies. This heterogeneity, added to other factors such as the biological sample and the analytical procedure used in each study, greatly makes the identification of universal HCC biomarkers difficult. Biomarkers are indicators that would be specific for HCC and not detected in premalignant liver disease, sensitive, enabling detection at an early stage when treatment is possible, and easily measurable by reproducible and minimally invasive tests. In addition to the exploratory phase aimed at the identification of potential biomarkers using reliable and reproducible assays, further studies are essential to validate the target proteins as biomarkers and assess how close they are to clinical application according to the criteria based on the systematic analysis of large cohorts of patients and controls to evaluate the capability to differentiate subjects with from those without cancer even at an early stage, to detect preclinical disease, and to allow surveillance of the relevant population (5). Therefore, advances on biomarker discovery and their application to the clinical management of HCC will require close collaboration and cooperation among researchers and clinicians to enhance the capabilities and reliability of proteomics tools and to define carefully all pre-analytical variables of the samples under study.

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