REVIEW ARTICLE

Oncoproteomics of hepatocellular carcinoma: from cancer markers’ discovery to functional pathways

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Abstract
Hepatocellular carcinoma (HCC) is a heterogeneous cancer with no promising treatment and remains one of the most prevailing and lethal malignancies in the world. Researchers in many biological areas now routinely identify and characterize protein markers by a mass spectrometry-based proteomic approach, a method that has been commonly used to discover diagnostic biomarkers for cancer detection. The proteomic research platforms span from the classical two-dimensional polyacrylamide gel electrophoresis (2-DE) to the latest Protein Chip or array technology, which are often integrated with the MALDI (matrix-assisted laser-desorption ionization), SELDI (surface-enhanced laser desorption/ionization) or tandem mass spectrometry (MS/MS). New advances on quantitative proteomic analysis (e.g. SILAC, ICAT, and ITRAQ) and multidimensional protein identification technology (MudPIT) have greatly enhanced the capability of proteomic methods to study the expressions, modifications and functions of protein markers. The present article reviews the latest proteomic development and discovery of biomarkers in HCC that may provide insights into the underlying mechanisms of hepatocarcinogenesis and the readiness of biomarkers for clinical uses.

Hepatocellular carcinoma (HCC) remains one of the leading causes of cancer death in Asia (1). Owing to its asymptomatic nature during the course of neoplastic development and malignant progression, HCC patients are usually presented in the advanced stage when first diagnosed. The late diagnosis of HCC significantly diminishes the options of curative treatments available and augments the chance of disease relapse. With the aim to allow early diagnosis of HCC, researchers in many biological areas now routinely identify and characterize molecular biomarkers for HCC development and progression by cDNA microarray and mass spectrometry (MS)-based proteomic approaches (2–5). Today, proteomics promises to be a powerful tool for cancer biomarker discovery (3, 6). Capitalizing on the newly identified biomarkers from cell lines and clinical samples (serum and tissues), we could develop in vitro clinical assays based on the diagnostic biomarkers set with high throughput and improved efficacy. This would greatly facilitate the implementation of surveillance programmes for high-risk populations, such as cirrhosis patients with chronic hepatitis B (HBV) or hepatitis C (HCV) infections, in order to better our patient management.

In addition to molecular diagnosis, certain proteomic markers are also found in association with tumour histology, aetiology and prognostic values (e.g. disease recurrence and overall survival). Indeed, tumour development invokes different physiologic changes that could elicit the invasion of normal defence mechanisms. Tumorigenesis is a multistep process involved in genetic alterations, which account mainly for the acquisition of invasiveness and metastatic ability based on different mechanistic pathways. The current paradigm of tumour development as described by Hanahan and Weinberg (7) involves the manifestation of six distinct physiological changes that dictates malignant growth and tumorigenesis: (a) growth signal autonomy, (b) growth-inhibitory signal prohibition, (c) resistance to apoptosis, (d) unrestricted replicative

*Equal contribution.
potential, (e) prolonged angiogenesis and (f) tissue invasion and metastasis. These changes occur in tumour cells and their surrounding stromal cells, which differ from normalcy via a series of premalignant states into invasive cancers. A better understanding and further insight into these tumorigenic pathways and pathogenic mechanisms will greatly facilitate our prioritization of cancer biomarkers and validation of therapeutic targets for HCC.

On-go – proteomics: from 2-DE to protein chip

In recent years, the combination of two-dimensional polyacrylamide gel electrophoresis (2-DE), mass spectrometry (MS) and bioinformatic tools has been widely used for proteomic research both in clinical medicine and biopharmaceutical industry (8, 9). Notably, this proteomic approach has been commonly applied in comparative proteome analysis for identification of disease-specific signatures in tumour cell lines, tissues and/or sera. The power of the 2-DE-based technology has been well received earlier by the research community for their usefulness of protein identification with post-translational modifications as well as protein–protein interaction networks. However, as many proteomic projects got underway, the limitations of this technology became gradually apparent. To date, alternative proteomic platforms are emerging to enhance the detection sensitivity and to increase the capacity and capability of the 2-DE-based approach. These include protein quantification by isotope-coded affinity tags (ICAT), the combination of accurate mass tags and Fourier transform ion cyclotron resonance, and protein expression profiling by surface-enhanced laser desorption/ionization (SELDI) (Fig. 1).

Fig. 1. Schematic workflow of various proteomic approaches and the protein information derived. The classic proteomic approach, coupled with mass spectrometry, provides identity and basic information, such as post-translational modifications and isoforms, of proteins. Recently, innovative proteomic methodology has been developed, revealing more information on the proteins, such as glycosylations and phosphorylations. This further extends the proteomic study of proteins towards functional aspects. In addition, the advantages and limitations of different approaches are shown. 2D-DIGE, two-dimensional fluorescence difference gel analysis; 2D-PAGE, two-dimensional polyacrylamide gel electrophoresis; HPLC, high-performance liquid chromatography; ICAT, isotope-coded affinity tag; ID, identity; IMAC, immobilized metal-ion affinity chromatography; ITRAQ, isobaric tags for relative and absolute quantification; LMW, low molecular weight; MOAC, metal oxide affinity chromatography; MS, mass spectrometry; MudPIT, multidimensional protein identification technology; SAX, strong anion exchange chromatography; SEC, size-exclusion chromatography; SILAC, stable isotope labelling with amino acids in cell culture; TiO2, titanium dioxide; TOF, time of flight.
Determination of changes in the relative or absolute concentration is fundamental to the discovery of valid biomarkers. ICAT is a protein quantitative-based approach that gives a more accurate, reproducible, and comprehensive result of protein expression patterns in disease vs. normal samples (10). Moreover, protein chip or array is another proteomic platform that provides a versatile method for global analysis of protein–protein interactions and functional or biochemical activities. Besides the SELDI, there are three major types of protein microarrays based on their surface features: functional protein microarray, antibody microarray, and reverse-phase microarrays (11–13). Applications of protein arrays include studies on protein–protein (14), protein–lipid (15), protein–nucleic acid (16), protein–drug (17), and antibody–antigen interactions (18). Detection of autoantibody reactivity is useful in immune profiling and biomarker discovery of tumour-associated antigens that may be useful in immunodiagnosing cancer and indicates the role of important biochemical pathways in disease (19). Both synthetic peptides and recombinant proteins can be printed on an array chip, and today we can use 1 µL of serum or plasma to profile 8000 defined proteins on a commercial protein microarray.

Another commonly used protein chip platform is the SELDI technology, which is based on the utilization of various chromatography principles or biological surfaces to capture proteins from complex biological mixtures according to their physicochemical properties. SELDI-TOF-MS essentially analyses serum samples to generate proteomic fingerprint patterns that may be unique to a particular disease state or malignancy. Recent studies where SELDI-TOF-MS were exploited in HCC include early diagnostic potential for HCC (20), serum profiling for detection of chronic hepatitis C to HCC and complement C3a potential biomarker identification in human chronic hepatitis C (21). These studies suggested that the proteomic pattern generated by SELDI protein chip system is important in disease diagnosis and in prognosis (22, 23). Besides, the newly derived ‘reverse capture’ autoantibody microarray has considerably enhanced the specificity in protein detection (19, 24) and further advanced the protein chip technology in clinical studies. Therefore, this allows identification of multiple biomarkers in a high-throughput manner as well as reliable profiling biomarker expression patterns.

Bioinformatics plays a crucial role in data analysis and interpretation for the generation of a recognizable pattern in disease diagnosis (20, 25–27). For feature selection and model establishment, receiver operating characteristic (ROC) can be first used to estimate the power of each peak in discriminating different groups and then pattern recognition by significant analysis of microarrays (SAM), two-way hierarchical clustering, artificial network (ANN), decision tree, and classification and regression tree (CART). Bioinformatic meta-analysis is a powerful tool to generate data clustering and pattern recognition, which may shed new insights into our understanding in disease mechanisms, diagnosis, treatment and prognosis.

Multidimensional protein identification technology (MudPIT), an emerging technique for the proteome analysis, is capable of analysing highly complex proteomic mixtures such as whole proteomes, organelles, and protein complexes. The MudPIT approach, which was developed by Yates and colleagues (28, 29), uses two chromatography separation steps interfaced back to back in a fused silica capillary in order to increase the resolution of proteome characterization by at least 10-fold. Peptides are trypsin digested and separated firstly by charge using a strong cation exchange resin and then by hydrophobicity using reverse-phase chromatography (30). The capillary channel is opened into a mass spectrometer that will typically be a tandem electrospray, and so peptides are ionized in the liquid phase and tandem mass spectrum will then be generated. The major advantage of MudPIT over the traditional 2D-GE is the process speed, as MudPIT couples 2D-LC with mass spectrometry and is an online process (31, 32). Although both high performance liquid chromatography/liquid chromatography–mass spectrometry mass spectrometry (HPLC/LC-MSMS) and MudPIT MS/MS are chromatographic based, however, MudPIT-MS/MS couples biphasic or triphasic microcapillary columns to HPLC, and can therefore be used to analyse complex peptide mixtures generated from biofluids, tissues, cells, organelles and detect proteins with post-translational modification (32). Moreover, the recent development of mass spectrometry has improved abilities of accurate mass measurements and resolution. For instance, Ion Cyclotron Resonance Fourier Transform (FT-ICR) mass spectrometer reduces the mass measurement to a frequency measurement and is therefore potentially capable of exceedingly high mass accuracy (33, 34). Orbitrap-MS operates by radically trapping ions about a central spindle electrode. Features of the Orbitrap include high mass resolution (up to 150,000), large space charge capacity, high mass accuracy (2–5 p.p.m.) and increased dynamic range (>10^5) (35, 36). Both these high-resolution spectrometers have greatly been applied in both proteomic and metabonomic.
applications for both peptide and metabolite characterization respectively.

Nevertheless, there are several advantages of 2-DE-based approaches that are currently unmatched by other proteomic methods. A number of modifications have been introduced into the 2-DE-based methodology to improve the sensitivity and accuracy. Current advancements include the following: (a) prefractionation of proteins by different chromatography or HPLC before 2-DE separation to reduce the complexity of the protein mixtures; (b) laser capture microdissection for sample preparation to enrich tumour cells from tissue specimens that are commonly used in clinical proteomic studies (37); and (c) fluorescent dye utilization to increase the sensitivity or visibility of protein detection in 2-DE gels. For instance, the fluorescence 2-D difference gel electrophoresis (DIGE) technology is a fluorescence prelabelling 2-DE-based approach (38). Protein mixtures are labelled with up to three fluorescent dyes known as CyDye DIGE fluoros. Quantitative protein analysis is achieved by exciting the different dyes at specified wavelengths and separated on the same 2-DE gel. With these recent advancements, 2-DE-based technology will remain an important tool for proteomic research in the near future.

Functional proteomics

Proteomics is used to study the complete set of proteins expressed in given cell lines, tissues, and biofluids, to elucidate the mechanisms behind HCC development and progression, and to discover new diagnostic and prognostic biomarkers and therapeutic targets (39). With the recent advent of new molecular and cellular techniques, many biomarkers related to tumour metastasis and recurrence and patient’s survival have been characterized with respect to their functionalities. Note that many of these cancer biomarkers are the key components in the regulatory pathways that govern the functional acquisition of tumour growth and invasion. While both the clinical tissues and cell lines are commonly used for cancer marker discovery, the diagnostic values of biomarkers identified in circulating body fluid (blood, urine) have drawn attention and increasing interest, owing to the easy access and accessibility from clinical subjects. Herein, we describe and summarize the HCC oncoproteomics with special reference to functional classifications of clinical biomarkers (those identified in HCC tissues and sera) over the past 5 years. We outline the common HCC signalling pathways or physiologic changes that may be involved in tumour development and associated with clinical prognosis. Given such information (as summarized in Table 1), researchers may exploit new biomarkers and speculate the potential role(s) or clinical values relating to HCC. The following sections focus on markers that have been reported in both the HCC tissues and serum samples and have been outlined according to the molecular, cellular or physiological functions.

Tissues

Acute phase reaction

Apolipoprotein E (ApoE), an acute phase-reactive protein, is one of the recently identified potential biomarkers in HCC (Table 1). Overexpression of ApoE has already been observed in a variety of cancers, which include brain (54), breast (55), ovarian (56) and prostate (57) cancers. ApoE has been shown to modulate immune function (inhibition of interleukin-2 production and lymphocyte proliferation) (58), inhibit tumour growth (59) and alter β-catenin distribution, inducing cell growth and migration (60). Although ApoE has been incriminated as a risk factor for many cancers, the role of ApoE in HCC is still speculative.

Ubiquitin-conjugating enzyme E2N, is another protein in this functional group that is found to be upregulated in both the HBV- and HCV-associated HCC tumours (40). The ubiquitin–proteasome system involves the attachment of ubiquitin to protein targets for processing by the 26S proteasome (61). Evidence also suggested that perturbations of the ubiquitin system were related to tumour progression through alternations in cell cycle and immune control (62).

Biotransformation

Proteins that belong to this functional group are mostly enzyme reductases that participate in glucose metabolism and osmoregulation. The aldo–keto reductase family is believed to play a protective role against toxic aldehydes derived from lipid peroxidation and steroidogenesis, which could affect cell growth/differentiation when accumulated (63). Li et al. (40) identified two members of the chlordecone reductase family, named aldo–keto reductase family 1, members C2 and C3, to be upregulated in HBV-related HCC tissues. Genes from the chlordecone reductase family were also found to be upregulated at the transcriptome level (64). Concordant results were obtained from the proteomic and transcriptomic expression profiles, indicating overexpression of the chlordecone reductase family in HCC. Another protein member in this group, aldo–keto reductase family
Table 1. Functional classification of hepatocellular carcinoma (HCC) biomarkers in proteomics studies

| Protein species | MW (kDa)/pI | HBV/HCV | Progn. values | Pattern in HCC | MS tools | Validation | References |
|----------------|-------------|---------|---------------|----------------|----------|------------|------------|
| **A. HCC tumour tissue** | | | | | | | |
| Acid–base balance | | | | | | | |
| Carbonic anhydrase I | 28.91/6.59 | HBV | Poor | M, L | Q, M, L (40, 41) |
| Carbonic anhydrase II | 28.75/6.63 | HBV | | L | (9) |
| Acute phase reaction | | | | | | | |
| α-1B glycoprotein | 54.27/5.58 | HCV | | M, L | (42) |
| Apolipoprotein E | 36.15/5.65 | HCV | | M, L | WB (42, 43) |
| Fibrinogen, α chain isoform | 70.23/8.23 | HBV | | M, L | (40) |
| Fibrinogen-like 1 precursor | 36.38/5.58 | HCV | | M, L | (42) |
| Serum amyloid P | 25.39/6.1 | HCV | | M, L | (42) |
| Ubiquitin carboxyl-terminal esterase L3 | 26.34/4.84 | HBV | | M, L | (40) |
| Ubiquitin-conjugating enzyme E2N | 17.18/6.13 | HBV | | M, L | (40) |
| Ubiquitin-specific protease 14 | 56.07/5.2 | HCV | | M, L | (42) |
| Amino acid metabolism | | | | | | | |
| 3-Hydroxyanthranilate 3,4-dioxygenase | 32.54/5.62 | HCV | | M, L | (42) |
| Adenosylhomocysteinase | 47.72/5.92 | HCV | Poor | M, L | (41, 42) |
| Aminocyclase | 45.86/5.77 | | M, L | | (9) |
| Aminocyclase 1 | 45.89/5.77 | HCV | Poor | M, Q | (41, 42) |
| Arginase 1 | 34.73/6.72 | HCV | Poor | M, L | WB | (41, 6) |
| Betaine-homocysteine methyltransferase | 44.97/6.4 | HCV | | M, L | (42) |
| Betaine-homocysteine S-methyltransferase | 44.87/6.4 | Well | | Q, M | (41) |
| C-1-Tetrahydrofolate synthase, cytoplasmic | 101.56/6.9 | Poor | | Q, M | (41) |
| Carbamoyl-phosphate synthase (ammonia) precursor | 164.83/6.28 | HBV | | M, L | (40) |
| Carbamoyl-phosphate synthetase 1, mitochondrial | 164.94/6.3 | HCV | | M, L | (42) |
| Catechol-O-methyltransferase isoform | 24.83/5.15 | HBV | | M, L | (40) |
| Dimethylarginine dimethylaminohydrolase 1 | 31.12/5.53 | HCV | | M, L | (42) |
| Formiminotransferase cyclodesaminase | 58.93/5.58 | HCV | Poor | M, L | (41, 42) |
| Glutamate synthase | 42.15/6.61 | HCV | | M, L | (40) |
| Glycine N-methyltransferase | 32.74/6.5 | Poor | | M, L | (41) |
| Hepatic peroxysomal alanine:glyoxylate aminotransferase | 39.78/6.85 | HBV | | M, L | (40) |
| Methionine adenosyltransferase | 37.54/6.9 | M | | L | (9) |
| Methionine adenosyltransferase 1 | 43.65/5.86 | HCV | | M, L | (42) |
| Methylenaldehyde dehydrogenase 1 | 83.16/4.4 | HCV | | M, L | (42) |
| Phenylalanine hydroxylase | 51.86/6.15 | HCV | | M, L | (42) |
| Pyrroline-5-carboxylate reductase 1 | 33.38/7.18 | HBV | | M, L | (40) |
| Transglutaminase C | 89.79/5.68 | HCV | | M, L | (42) |
| Anti-oncogene, differentiation | | | | | | | |
| Human thioredoxin mutant with Cys 73 replaced by Ser (reduced form) | 11.72/4.82 | HBV | | M, L | (40) |
| Fumarate hydratase precursor, mitochondrial | 50.39/7.23 | HBV | | M, L | (40) |
| Hccl-2 | 40.7/5.52 | Poor | | M | PCR (44) |
| Nm 23 | 17.15/5.83 | HCV | | M, L | (5) |
| N-myc downstream-regulated gene 1 protein | 42.84/5.49 | HCV | | M, L | (42) |
| PKCp-interacting protein PICOT | 37.69/5.31 | HBV | | M, L | (40) |
| Biotransformation | | | | | | | |
| Aldo–keo reductase family 1, member B10 | 36.02/7.1 | Poor | | M, Q | (41) |
| Aldo–keo reductase family 1, member C2 | 36.74/7.13 | HBV | | M, L | (40) |
| Aldo–keo reductase family 1, member C3 | 37.23/8.06 | HBV | | M, L | (40) |
| Aldose reductase | 35.8/6.5 | | M | IHC (45) |
| Aryl sulphotransferase ST1A3 | 34.3/6.16 | HBV | | M, L | (40) |

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| Protein species | MW (kDa)/pI | HBV/HCV | Prognos. values | Pattern in HCC | MS tools | Validation | References |
|-----------------|-------------|---------|-----------------|---------------|----------|------------|------------|
| Glutathione peroxidase | 22.23/6.15 | HBV | ↓ | M, L | (40) |
| Manganese superoxide dismutase | 24.71/8.35 | HBV | ↓ | M, L | (40) |
| Superoxide dismutase 1 | 15.94/5.7 | HBV | ↓ | M, L | (40) |
| Carbohydrate metabolism | | | | | | | |
| Aldolase B | 39.85/8.28 | HBV | ↓ | M, L | (40) |
| α-Enolase | 47.17/7.01 | HCV | Poor, VI, size | L WB, IHC | (46) |
| Enolase-1 | 47.48/7.01 | HBV | ↑ | M, L | (40) |
| Fructose-1,6-biphosphatase | 36.81/6.54 | HCV | ↓ | M, L | (42) |
| Fructose-1,6-bisphosphatase | 36.81/6.5 | Poor | ↓ | M, Q | (41) |
| Fructose-bisphosphatase | 37.2/6.54 | HBV | ↓ | M, L | (41) |
| Fructose-bisphosphate aldolase | 39.44/8 | ↓ | L | (9) |
| Fructose-bisphosphate aldolase B | 39.47/8 | Poor | ↓ | Q, M | (41) |
| Fumarate hydratase, mitochondrial precursor | 54.64/8.9 | HBV | Well | ↓ | Q, M | (41) |
| Catabolism | | | | | | | |
| 4-Hydroxyphenylpyruvate dioxygenase | 44.8/6.5 | Poor | ↓ | Q, M | (41) |
| Acetyl-CoA acetyltransferase, mitochondrial precursor | 45.2/9 | Poor | ↓ | Q, M | (41) |
| Fumarase | 46.37/6.5 | Poor | ↓ | Q, M | (41) |
| Glycine amidinotransferase, mitochondrial precursor | 48.46/8.2 | Poor | ↓ | Q, M | (41) |
| Homogentisate 1,2-dioxygenase | 49.97/6.5 | Poor | ↓ | Q, M | (41) |
| Isocitrate dehydrogenase, cytoplasmic | 46.63/6.2 | Well | ↓ | Q, M | (41) |
| Pyridoxine 5'-phosphate oxidase | 29.99/6.6 | Poor | ↓ | Q, M | (41) |
| Cell cycle | | | | | | | |
| 14-3-3 Protein γ | 28.15/4.8 | ↑ | L | WB | (9) |
| Cell proliferation and growth | | | | | | | |
| Cofilin 2 isoform 1 | 18.84/7.66 | HBV | ↑ | M, L | (40) |
| Mitochondrial ribosomal protein L12 | 21.38/9.05 | HBV | ↑ | M, L | (40) |
| Nucleophosmin | 28.5/5.56 | HBV | ↓ | M, L | (40) |
| Phosphinositol 4-phosphate adaptor protein-2 | 57.65/5.23 | HBV | ↑ | M, L | (40) |
| Proliferating cell nuclear antigen | 28.77/4.57 | HBV | ↑ | M, L | WB | (40) |
| Stathmin 1 | 17.3/5.76 | HBV | ↑ | M, L | WB | (40) |
| Chaperones | | | | | | | |
| FK506-binding protein 4 | 51.8/5.35 | HBV | ↑ | M, L | (40, 42) |
| Glucose-regulated protein 75 | 73.68/5.87 | HBV | ↑ | M, L | (40) |
| Glucose-regulated protein 78 | 72.3/5.1 | VI | ↑ | Q | WB, IHC | (47) |
| GrpE-like protein cochaperone | 24.28/8.24 | HBV | ↑ | M, L | (40) |
| Heat shock protein 27 | 22.8/6.0 | AFP | ↑ | M, L | WB, IHC | (9, 47) |
| Heat shock protein 60 | 61.05/5.7 | HCV | ↑ | M, L | (42) |
| Heat shock protein 70 | 70/5.5 | ↑ | Q, L | WB, IHC | (9, 47) |
| Heat shock protein 70 protein 5 | 72.45/0.7 | HBV | ↑ | M, L | (40) |
| Heat shock protein 70 protein 6 | 71.03/5.81 | HCV | ↑ | M, L | (42) |
| HSPC231 | 11.88/5.48 | HBV | ↑ | M, L | (40) |
| Mitochondrial heat shock protein 75 | 74.02/5.97 | HBV | ↑ | M, L | (40) |
Table 1. Continued.

| Protein species | MW (kDa)/pl | HBV/HCV | Prognos. values | Pattern in HCC | MS tools | Validation | References |
|----------------|-------------|---------|-----------------|----------------|----------|------------|------------|
| Stress-induced phosphoprotein 1 | 62.64/6.4 | HBV | ↑ | M, L | (40) |
| Cytoskeleton and extracellular matrix/mechanism | | | | | | | |
| Actin γ1 | 41.77/5.31 | | ↑ | L | (9) |
| Actin-related protein 2/3 complex subunit 5 | 16.18/5.47 | | ↓ | L | (9) |
| Class IV b tubulin | 49.72/4.82 | | ↑ | L | (9) |
| Collagen α 1 | 108.64/5.26 | HCV | ↓ | M, L | (42) |
| Cytokeratin 8 | 53.56/5.52 | HCV | ↓ | M, L | (42) |
| Cytokeratin 10 | 59.52/5.1 | | Poor | Q, M | (41) |
| Cytokeratin 18 | 47.33/5.27 | HCV | ↓ | M, L | (42) |
| Cytoplasmic dynein intermediate chain 2C | 68.35/5.2 | HCV | ↑ | M, L | (42) |
| Macrophage capping protein (actin-3) | 38.5/5.82 | HCV | ↓ | M, L | (42) |
| Mutant β-actin | 42.13/5.22 | HBV | ↓ | M, L | (40) |
| Profilin, chain A | 14.84/8.46 | | ↑ | L | (9) |
| Smoothelin | 99.52/9.16 | HCV | ↓ | M | (6) |
| TPMsk3 (tropomyosin fragment) | 28.79/4.72 | | ↑ | L | (9) |
| β-Tubulin cofactor A | 32.85/4.66 | HCV | ↓ | M | (6) |
| Villin-2 | 69.41/5.94 | HCV | ↓ | M, L | (42) |
| Vimentin | 53.59/5.06 | HBV | ↓ | M, L | (40) |
| Detoxication, oxidoreduction | | | | | | | |
| Alcohol sulphotransferase | 33.78/5.71 | HCV | ↓ | M, L | (42) |
| Aldehyde dehydrogenase | 54.86/6.3 | HCV | ↓ | M, L | (42) |
| Aldehyde dehydrogenase 1A1 | 55.45/6.3 | HBV | ↑ | M, L | (40) |
| Aldehyde dehydrogenase 3 | 50.34/5.99 | HCV | ↑ | M, L | (42) |
| Aldehyde dehydrogenase 5 | 57.31/6.54 | HCV | ↓ | M, L | (42) |
| Aldehyde dehydrogenase 9 | 53.8/5.69 | HCV | ↓ | M, L | (42) |
| Aldehyde dehydrogenase B | 56.38/6.63 | HCV | ↓ | M, L | (42) |
| Aldehyde dehydrogenase, mitochondrial precursor | 56.39/6.6 | Poor | ↓ | Q, M | (41) |
| Antioxidant protein 2 | 25.04/6 | Poor | ↓ | Q, M | (42) |
| α-1-Antitrypsin precursor | 46.74/5.4 | Poor | ↓ | Q, M | (41) |
| Catalase | 59.63/7 | Poor | ↓ | Q, M | (41) |
| Cytochrome b5 | 15.33/4.88 | HCV | ↓ | M, L | (42) |
| Epoxide hydrolase 2 | 62.62/5.91 | HCV | ↓ | M, L | (42) |
| Fibrinogen γ chain precursor | 55.28/5.2 | HCV | ↓ | M, L | (42) |
| Flavin reductase | 32.85/4.66 | HCV | ↓ | M, L | (42) |
| Glutamate-cysteine ligase regulatory protein | 30.73/5.7 | HCV | ↑ | M, L | (42) |
| Glutathione-S-transferase | 27.57/6.24 | HCV | ↓ | M, L | (42) |
| Glutathione-S-transferase A1 | 25.63/8.9 | Poor | ↓ | Q, M | (41) |
| Glutathione-S-transferase omega 1 | 23.33/6.75 | | ↑ | L | (9) |
| Glutathione synthetase | 52.39/5.67 | HCV | ↓ | M, L | (42) |
| Heat shock protein 70 9B | 73.73/6.03 | HCV | ↑ | M, L | (42) |
| Haeme-binding protein | 21.1/5.7 | HCV | ↑ | M, L | (42) |
| Human aldose reductase-like protein 1 | 36/7.4 | | ↑ | M | IHC |
| Liver carboxyesterase | 62.52/6.15 | HCV | ↓ | M, L | (42) |
| MAWD-binding protein | 31.79/6.06 | HBV/HCV | Poor | Q, M | (40, 42) |
| NADH dehydrogenase (ubiquinone) Fe-S protein | 79.47/5.89 | HCV | ↓ | M, L | (42) |
| Peroxiredoxin 1 | 22.11/8.27 | HCV | ↓ | M, L | (42) |
| Peroxiredoxin 2 | 27.69/7.68 | HCV | ↓ | M, L | (42) |
| Proteasome β-chain | 2.79/4.37 | HCV | ↓ | M, L | (42) |
| Selenium-binding protein 1 | 52.31/6.13 | HCV | ↓ | M, L | (42) |
| Thioredoxin | 11.74/4.82 | HCV | ↑ | M, L | (42, 9) |
| Thiosulphate sulphurtransferase | 33.64/6.77 | HBV/HCV | ↓ | M, L | (40, 42) |
| Transaldolase | 37.63/7.67 | HCV | ↑ | M, L | (42) |
Table 1. Continued.

| Protein species | MW (kDa)/pI | HBV/HCV | Prognos. values | Pattern in HCC | MS tools | Validation | References |
|----------------|------------|---------|----------------|----------------|----------|------------|------------|
| **Energy metabolism** | | | | | | | |
| ATP synthase β chain, mitochondrial precursor | 56.56/5.26 | HCV | ↓ | M, L | | (42) |
| Electron transfer flavoprotein, α polypeptide | 35.08/8.62 | HBV | ↓ | M, L | | (40) |
| Electron transfer flavoprotein α subunit mitochondrial precursor | 35.08/8.6 | Poor | ↓ | Q, M | | (41) |
| H⁺-transporting two-sector ATPase β chain precursor, mitochondrial | 56.53/5.26 | HBV | ↓ | M, L | | (40) |
| Liver-type aldolase | 39.47/8.01 | HCV | ↓ | M | WB | (6) |
| Succinate dehydrogenase | 72.69/7.06 | HCV | ↓ | M, L | | (42) |
| **Ethanol catabolism** | | | | | | | |
| Alcohol dehydrogenase | 36.42/6.34 | Poor | ↓ | L, M, Q | | (9, 41) |
| **Fatty acid metabolism** | | | | | | | |
| 2,4-Dienoyl CoA reductase 1 precursor | 36.33/9.35 | HBV | ↓ | M, L | | (40) |
| 3-Hydroxy-3-methylglutaryl-coenzyme A synthase 1 | 57.35/5.33 | HCV | ↑ | M, L | | (42) |
| 3-Hydroxy-3-methylglutaryl-coenzyme A synthase 2 | 56.64/8.4 | HCV | ↓ | M, L | | (42) |
| Acyl-CoA dehydrogenase, short chain specific, mitochondrial precursor | 44.3/8.1 | Poor | ↓ | Q, M | | (41) |
| Enoyl-CoA hydratase | 31.39/8.34 | HCV | Well | Q, M | | (6, 41) |
| Fatty acid-binding protein | 14.12/6.6 | | | L | | (9) |
| Fatty acid-binding protein 1 | 14.2/6.6 | HCV | ↓ | M, L | | (42) |
| Glycerol-3-phosphate dehydrogenase, cytoplasmic | 37.59/5.8 | Poor | ↓ | Q, M | | (41) |
| Glycerol-3-phosphate dehydrogenase 1 | 37.57/5.81 | HCV | ↓ | M, L | | (42) |
| α-Crystallin | 34.3/5.95 | HBV/HCV | ↓ | M, L | | (40, 42) |
| Leukotriene A4 hydrolase (Grp 94) | 69.29/5.8 | HCV | ↓ | M, L | | (42) |
| Mitochondrial short-chain enoyl-coenzyme A hydratase 1 precursor | 31.37/8.34 | HCV | ↓ | M, L | | (42) |
| **Immunity** | | | | | | | |
| Chain A, Cypa complexed with hagpia | 17.88/7.82 | HBV | ↑ | M, L | | (40) |
| Haptoglobin precursor | 38.43/6.13 | | ↓ | L | | (9) |
| IgM rheumatoid factor RF-DI3, variable heavy chain | 14.08/9.39 | HBV | ↑ | M, L | | (40) |
| Serologically defined colon CA-10 | 42.81/5.51 | HBV | ↑ | M, L | | (40) |
| **Iron buffering** | | | | | | | |
| Ferritin light chain | 20.01/5.51 | HCV | ↓ | Q, M | WB, IHC | (6, 9) |
| **Metastasis and apoptosis** | | | | | | | |
| Lamin B1 | 66.41/5.11 | HCV | ↑ | M, L | | (42) |
| Laminin receptor 1 | 32.95/4.79 | HBV | ↑ | M, L | | (40) |
| Non-metastatic cells 1 protein | 17.15/5.83 | HBV | ↑ | M, L | | (40) |
| P47 | 40.55/5.03 | HBV | ↑ | M, L | | (40) |
| Serine (or cysteine) proteinase inhibitor, clade B (ovalbumin), member 9 | 43/5.61 | HBV | ↓ | M, L | | (40) |
| **Nitrogen metabolism** | | | | | | | |
| Glutamate dehydrogenase | 61.36/7.66 | | ↓ | L | | (9) |
| **Nucleotide, nucleic acid, cofactors, and vitamins metabolism** | | | | | | | |
| ADP-sugar pyrophosphatase YSA1H | 24.6/4.87 | HBV | ↑ | M, L | | (40) |
| Guanine deaminase | 51/5.44 | HCV | ↓ | M, L | | (42) |
| Regucalcin | 33.25/5.89 | HCV | ↓ | M, L | | (42) |
| Senescence marker protein-30 | 33.25/5.9 | Poor | ↓ | Q, M | | (41) |
| Nicotinamide N-methyltransferase | 29.56/5.56 | HCV | ↓ | M, L | | (9, 42) |
| β-Ureidopropionase | 43.65/6.09 | HBV | ↓ | Q, M | | (40, 41) |
# Table 1. Continued.

| Protein species | MW (kDa)/pl | HBV/HCV | Prognos. values | Pattern in HCC | MS tools | Validation | References |
|-----------------|-------------|---------|-----------------|----------------|---------|------------|------------|
| Others          |             |         |                 |                |         |            |            |
| Albumin         | 69.37/5.92  | HCV     |                |                |         |            | (6)        |
| CGI-150 protein | 55.01/8.99  | HCV     |                |                |         |            | (42)       |
| Differentially expressed in FDCP (mouse homolog) 6 | 39.58/6.53 | HBV |                |                | M, L   |            | (40)       |
| Perchloric-acid-soluble translational inhibitor p14.5 | 14.54/8.74 | HBV |                |                | M, L   |            | (40)       |
| S-adenosylmethionine synthase α and β forms | 43.65/5.7  | Poor |                |                | Q, M   |            | (41)       |
| SH3-binding glutamate-rich protein | 12.77/5.22 | HBV |                |                | M, L   |            | (40)       |
| Wnt-1           | 40.98/9.28  | HBV/HCV | ↑               |                | M, L   | WB         | (48)       |
| Peptidase/proteases/protease inhibitors |             |         |                 |                |         |            |            |
| Cathepsin A     | 54.47/6.16  | HBV     | ↑               |                | M, L   |            | (40)       |
| Cathepsin D     | 44.55/6.1   | HCV     | ↓               |                | M, L   |            | (42)       |
| Cathepsin D, chain A | 10.67/5.65 | HCV | ↓               |                | L      |            | (9)        |
| chain B         | 26.23/5.31  |         |                 |                |         |            |            |
| N-acetylaminoacyl-peptide hydrolase | 81.22/5.29 | HCV |                |                | M, L   |            | (42)       |
| Phosphatidylethanolamine-binding protein | 20.93/7.4  | Poor |                |                | Q, M   |            | (41)       |
| Pro tease inhibitor 2 | 42.74/5.9  | HCV     | ↑               |                | M, L   |            | (42)       |
| Tripeptidyl-peptidase I | 61.23/5.97 | HCV | ↑               |                | M, L   |            | (42)       |
| Protein metabolism |             |         |                 |                |         |            |            |
| Elongation factor Tu, mitochondrial precursor | 49.54/7.3  | Poor |                |                | Q, M   |            | (41)       |
| Peptidylprolyl isomerase | 18/7.68   | ↑       |                |                | L      |            | (9)        |
| Peptidylprolyl isomerase A | 18.01/7.68 | HBV | ↑               |                | M, L   |            | (40)       |
| Ribonucleoprotein, transcription |             |         |                 |                |         |            |            |
| 60 Far upstream element-binding protein | 67.69/7.18 | HBV | ↑               |                | M, L   |            | (40)       |
| DNA directed RNA polymerase II | 31.76/4.79 | HBV | ↑               |                | M, L   |            | (40)       |
| Eukaryotic translation elongation factor 1 α 1 | 50.45/9.1  | HBV     | ↓               |                | M, L   |            | (40)       |
| Eukaryotic translation initiation factor 5A | 16.83/5.07 | HCV |                |                | M, L   |            | (42)       |
| Heterogeneous nuclear ribonucleoprotein K | 50.98/5.39 | HCV | ↑               |                | M, L   |            | (42)       |
| HNRPC protein | 33.64/9.9   | HBV     | ↑               |                | M, L   |            | (40)       |
| Nucleophosmin | 32.58/4.64  | HCV     | ↑               |                | M, L   |            | (42)       |
| TATA-binding protein interacting protein 49 kDa | 50.23/6.02 | HBV | ↑               |                | M, L   |            | (40)       |
| Transcription factor E2F-4 | 44.54/4.66 | HBV | ↓               |                | M, L   |            | (40)       |
| Steroid metabolism |             |         |                 |                |         |            |            |
| 3-Hydroxysteroid dehydrogenase | 37.07/6.71 | ↑       |                |                | L      |            | (9)        |
| Translation |             |         |                 |                |         |            |            |
| Eukaryotic translation elongation factor 1 delta isoform 2 | 31.22/4.9 | HBV | ↑               |                | M, L   |            | (40)       |
| Eukaryotic translation elongation factor 3 subunit 4 | 35.61/5.78 | HBV | ↑               |                | M, L   |            | (40)       |
| Ribosomal protein P0 | 34.42/5.71 | HBV | ↑               |                | M, L   |            | (40)       |
| Splicing factor, arginine/serine-rich 1 | 27.75/10.37 | HBV | ↑               |                | M, L   |            | (40)       |
| Transport |             |         |                 |                |         |            |            |
| Annexin IV | 36.08/5.84  | HBV     | ↓               |                | M, L   |            | (42)       |
| Annexin V | 35.84/4.98  | HBV     | ↓               |                | M, L   |            | (40)       |
| Annexin VI | 75.87/5.42  | HBV     | ↓               |                | M, L   |            | (42)       |
| Chloride intracellular channel 1 | 26.92/5.09 | HCV | ↑               |                | M, L   | WB         | (42)       |
| Human rab GDI | 50.66/5.94  | HCV     | ↑               |                | M, L   |            | (42)       |
1, member B10 had been found by the Liang group (41) to be overexpressed in poorly differentiated HCC.

**Carbohydrate metabolism**

Fructose-bisphosphate aldolase B is a liver-specific enzyme for glucose and fructose metabolism. A functional change of this enzyme may effect changes from metabolism to proliferation that accelerate the metastasis of HCC. For instance, fructose-1,6-bisphosphate aldolase in this group is a glycolytic enzyme that catalyses the reversible conversion of fructose-1,6-bisphosphate to glyceraldehyde 3-phosphate and dihydroxyacetone phosphate, whereas fructose-1,6-bisphosphatase catalyses the hydrolysis of fructose-1,6-bisphosphate to fructose-6-phosphate and inorganic phosphate. Both these enzymes are predominant in the human liver and muscle. Liang et al. (41) reported a relatively

### Table 1. Continued.

| Protein species | MW (kDa)/pI | HBV/HCV | Prognos. values | Pattern in HCC | MS tools | Validation | References |
|-----------------|-------------|---------|----------------|---------------|----------|------------|------------|
| Interferon-induced viral resistance protein MxA | 75.58/5.65 | HCV | ↓ M, L | (42) |
| Non-specific lipid-transfer protein, mitochondrial precursor | 58.99/6.4 | Poor | ↓ Q, M | (41) |
| Rho GDP dissociation inhibitor β | 23.02/4.96 | HBV/HCV | ↓ M, L | (40, 42) |
| Serotransferrin precursor | 77.05/6.8 | Well | ↓ Q, M | (41) |
| Sterol carrier protein 2-related form | 58.85/6.15 | HCV | ↓ M, L | (42) |
| Transferrin receptor | 84.9/6.18 | ↑ M, L | (43) |
| Vesicle amine transport protein | 41.92/5.88 | HCV | ↓ M, L | (42) |

### B. Serum

#### Acute phase reaction

| Apolipoprotein A1 isoform | 30.78/5.56 | ↓ M | (49) |

#### Chaperones

| Heat shock protein 27 | 22.8/5.98 | ↑ Q WB | (50) |

#### Detoxification and oxidoreduction

| α1 antitrypsin | 46.7/5.37 | ↑ Q | (50) |
| Ceruloplasmin | 157.2/5.27 | HBV | ↑ Q | (50) |
| Fatty acid metabolism | | | |
| Clusterin | 37.2/4.89 | ↑ Q | (50) |

#### Growth factor and cytokines

| Brain-derived neurotrophic factor (BDNF) | 27/– | ↑ R | M, Q PCR, WB | (51) |

#### Immunity

| Autoantibodies, calreticulin | –/– | ↑ M | (52) |
| Autoantibodies, Cytokeratin 8 | –/– | ↑ M | (52) |
| Autoantibodies, Fv-1,6-ATP synthase β-subunit | –/– | ↑ M | (52) |
| Autoantibodies, Nucleotide diphosphate kinase A | –/– | ↑ Q | (52) |

| Complement C3a | 53.86/6.2 | HCV | ↑ S, L CI, WB | (21) |
| Complement C3 fragment | 41.49/4.96 | HBV | ↓ M | (49) |
| Haptoglobin α2 chain | 45.2/6.13 | ↑ Q | (50) |

#### Others

| α-Fetoprotein | 47.3/5.97 | ↑ Q | (50) |

#### Transport

| Transferrin | 76.9/6.81 | HBV | ↓ Q | (50) |
| Transthyretin | 15.8/5.52 | ↓ Q | (50) |
| Vitronectin precursor, C-terminal fragment | 8.9/– | Size | S PCR | (53) |

Cl, chip immunoassay; HBV, hepatitis B virus; HCV, hepatitis C virus; R, recurrence; IHC, immunohistochemistry; LC, liquid chromatography; MALDI, matrix-assisted laser desorption/ionization-time of flight; MS, mass spectrometry; MS/MS, tandem mass spectrometry; MW, molecular weight; RT-PCR, reverse transcription-polymerase chain reaction; SELDI, surface-enhanced laser desorption/ionization; WB, Western blot.

Mass spectrometry methods used for protein identification: L, LC-MS/MS; M, MALDI-ToF/MS; Q, tandem MS/MS; S, SELDI-ToF.
low-expression protein level in poorly differentiated HCC, and parallel results were observed at the transcriptomic level. Kim et al. (65) suggested that downregulation of fructose-bisphosphate aldolase B in HBV-associated HCC was mainly attributed to the diminished liver-enriched transcription factors. Other groups also reported similar findings (66, 67).

**Cell proliferation and growth**

Two proteins in this functional group, proliferating cell nuclear antigen (PCNA) and Stathmin 1, were overexpressed in HCC (40). As an index of cell proliferation, overexpression of PCNA is used as a reliable marker for the assessment of tumour progress, premalignant evolution and clinical prognosis of patients with various malignancies. Chromosomal localization of the PCNA gene, 20pter-p12, was mapped within a cancer-related chromosome region 20pter-q12 (serologically defined breast cancer antigen 84). Stathmin 1 is a cytoplasmic tubulin-binding phosphoprotein that is known to play an important role in cellular proliferation (68, 69). It functions as a depolymerizer of microtubules and is known to be overexpressed in ovarian cancer and breast cancer. In addition, the Stathmin 1 mRNA level was also found to be up-regulated in HCC (70). Chromosomal localization of the Stathmin 1 gene, 1p36.1-p35, is associated with three cancer-related chromosome regions: 1p36 (breast and ductal cancer), 1p36 (prostate cancer–brain cancer susceptibility), and 1p36.11 (upregulated in liver cancer 1). This suggests that a link between the genomic and proteomic data may provide a better insight into the disease pathology and metastasis.

**Transport**

Blanc et al. (42) have found that chloride intracellular channel 1 (CLIC-1) was upregulated in a majority of HCV-HCC-related patients. This protein is a member of the class of chloride ion channels. The CLIC family proteins are involved in a wide variety of cellular functions, which include the regulation of anion secretion, cell division, and apoptosis (71). More importantly, the role of CLIC-1 in hepatocarcinogenesis has recently been reported by a few studies (42, 72). Another transport protein that was reported to be upregulated in HCC is the transferrin receptor protein, TfR (43). This protein is responsible for iron transport and storage, mainly for iron homeostasis. It has been well anticipated that excess iron in the hepatocyte is associated with increased risk for HCC (73). In Park’s report, TfR was shown to be overexpressed in HCC tissues (43). However, there is no direct correlation between mRNA and protein expression of this protein and no difference in the relative mRNA expression levels of TfR between normal and HCC tumours. This result is consistent with the previous report by Yamashita et al. (74).

**Others**

Different disease-related biomarkers could be grouped according to their functions. Biomarkers identified recently are mainly classified into three main functional groups, which include (a) amino acid metabolism, (b) cytoskeleton and extracellular matrix, (c) detoxication and oxidoreduction. However, most of the biomarkers in these groups have not yet been fully validated. Other functional groups including acid–base balance, ethanol catabolism, iron buffering, steroid metabolism, and nitrogen metabolism are comparatively smaller groups with less biomarkers identified for HCC.

**Serum**

**Growth factors and cytokines**

Brain-derived neurotrophic factor (BDNF) and glypican-3 are the two fervent serum biomarkers in this group. BDNF is a member of the nerve growth factor family; it was found to be gradually upregulated during tumour development in both HCC serum and tissues (51, 75). A comparatively low expression of this protein was also found in the adjacent nontumourous tissues and cirrhotic liver tissues. Re-elevated expression of BDNF was also found in an orthotopic rat HCC model during HCC recurrence. This clearly suggested that BDNF is involved in both HCC development and recurrence. Further confirmation of BDNF’s role in hepatocarcinogenesis was presented in tumour cell lines of HCC with elevated protein expression but not in normal cell lines. Glypican-3 (GPC3) is a member of heparin sulphate proteoglycans, which plays a role in cell growth, differentiation, and migration (76). GPC3 has been proposed as markers for HCC diagnosis (77). Overexpression of GPC3 mRNA and protein levels were detected in the serum and tissues of HCC patients (78) but not in normal hepatocytes and nonmalignant liver disease. More importantly, the NH2 terminal portion [(soluble GPC3 (sGPC3)] is cleaved between Arg358 and Ser359 of GPC3 and that of sGPC3 can be specifically detected in the sera of HCC patients. Combined use of both the GPC3 and α-fetoprotein (AFP) biomarkers was shown...
to improve significantly the diagnostic test sensitivity of HCC (79).

**Autoantibodies and immune-related proteins**

Le Naour *et al.* (52) suggested that a distinct repertoire of autoantibodies was associated with HCC that may have utility in the early diagnosis of HCC among high-risk subjects with chronic hepatitis. Calreticulin and a novel truncated form of calreticulin (Crt32) were found to induce autoantibodies in HCC patients. First, calreticulin was upregulated in HCC tumour tissue as compared with the non-tumour counterpart. It is a component of major histocompatibility complex I peptide-loading complex, and there are elevated levels of calreticulin in the nuclear matrix fraction of HCC but not in nonmalignant liver tissue (80). In the same study, cytokeratin 8 (CK8) and F1-ATP synthase β-subunit autoantibodies were also found in patients with HCC. Cancer cells are known to secrete CK8-containing protein complexes in *vitro* and in *vivo* (81). Many cell types express only one acidic (Type I) CK and one neutral-based (Type II) CK. For example, both CK8 and CK18 were found to be upregulated in hepatocytes of HCC patients (52).

Complement C3a is another biomarker that was found to be overexpressed in patients with chronic hepatitis C and HCV-related HCC (21). It has been recently identified by SELDI-TOF MS profiling analysis and the protein level is confirmed by PS20 chip immunoassay and Western blotting. Complement components are important mediators of inflammation and contribute to the regulation of the immune response. In cancer patients, complement activation with subsequent deposition of complement components on tumour tissue has been demonstrated (82). In addition, C3a, which is a proteolytic fragment of C3, has been reported as a potent inflammatory mediator of innate immune response, and contributes essentially to the early priming stages of hepatocyte regeneration after toxic injury and partial hepatectomy (83). However, Lee *et al.* (21) suggested that C3a was upregulated only in HCV-related HCC, but not in HBV-related HCC. It is well known that the molecular hepatocarcinogenesis might be different in the context of HBV and HCV infections, yielding a difference in C3a levels in HCC patients.

**Chaperones**

Heat shock protein (HSP) 27 has recently been identified as a potential biomarker for HCC by tissue and serum proteome analysis and the result was validated by Western blot (50). HSP27 is a stress-inducible cytosolic protein that is ubiquitous in many normal tissues. Recent studies, however, have shown that HSP27 may play important roles in thermo-tolerance, cellular proliferation and apoptosis, oestrogen response and molecular chaperoning (84, 85). HSP27 is not a specific tumour marker for HCC, as overexpression of this protein has been reported in many kinds of tumour tissues, which include brain tumour and breast cancer (86, 87). Further studies on the molecular mechanisms of HSP27 in HCC carcinogenesis are necessary.

**Detoxification and oxidoreduction**

α1 Antitrypsin (α1AT) and ceruloplasmin (Cp) are the two biomarkers belonging to the detoxification and oxidoreduction, and elevated levels were found in HCC sera (50). Liver disease with α1AT deficiency is caused by a gain of toxic function mechanism engendered by the accumulation of a mutant glycoprotein in the endoplasmic reticulum. It is the most common cause of metabolic paediatric liver disease and is one of the potent inhibitors of proteolytic enzymes naturally existing in serum. α1AT is considered to be a useful tumour marker for HCC (88) and studies have suggested that there is an increased level of this protein in HCC patients (89, 90). Cp is an acute phase protein, and increases in expression during inflammation. Pousset *et al.* (91) suggested that there was an increased level of Cp in the serum of transgenic mice developing HCC. Moderately increased levels of Cp have been reported to occur in human HCC, and increased plasma Cp concentrations were related to the more rapidly progressing tumours (92).

**Others**

Other functional groups with potential serum biomarkers for HCC include acute phase reaction, fatty acid metabolism and transport. Identification of biomarkers from the serum is comparatively more difficult than in tissue owing to the complex nature of serum. At present, different methodologies are used to identify serum biomarkers in HCC patients but often there are problems of reproducibility of the findings in other laboratories.

**HCC signalling pathways**

Phenotypic and molecular abnormalities accompanied by liver tumorigenesis often lead to reversion of cell adhesion functions and dysregulation of cellular
mechanisms. These observations obviously do not usually come alone without good causes. Indeed, these events occur because of abrupt alterations in the morphological features of the malignant cells owing to viral hepatitis infection or acute damage inflicted by toxicants or carcinogens (93). During these processes, signalling pathways vary when subjected to those stimuli, resulting in the undesirable phenomenon and alteration in cell physiology that collectively dictate malignant growth. Some of the HCC signalling pathways are known to be involved in the acquired capabilities of cancer and as such are the mitogen-activated protein kinase (MAPK) and ubiquitin/proteasome degradation pathways (94). In addition, specialized pathways associated with Wnt and Hedgehog are described as one of those factors triggering the malignant outcomes owing to liver disorder (94, 95). However, not all of them can be readily detected or identified using proteomic approaches and there are upfront limitations in finding biomarker-related pathways. In fact, it is believed that phosphorylation and dephosphorylation of some HCC pathway components can master the process and promote HCC pathogenesis. Moreover, characterization of protein phosphorylation sites is essential for the understanding of protein function and regulation in HCC, and is also an important part of many proteomic studies. To be able to identify phosphoproteins by mass spectrometry, isolation and enrichment are necessary prior to MS analysis (96). There are now commercially available tools for detection, isolation and quantification of phosphoprotein, kinases, and phosphatases. Besides, the application of chemical, metabolic or enzymatic incorporation of stable isotopes into phosphopeptides or phosphoproteins provides a platform to quantify the relative changes of phosphorylation events. Indeed, the interrelationship between HCC signalling pathways and phosphorylation events will continue to be an analytical challenge for proteomics for many years to come. Given below are the outlined pathways that are related to HCC and can be broadly classified into one or more of the acquired capabilities towards tumour development.

**HSP/stress response signalling**

Several members of the HSP family have been found to be closely correlated with different aspects of HCC progression by the proteomic profiling approach (40, 42, 47) (Table 1). These results postulate that heat shock conditions or stress inductions are possible mediators in HCC development. Under environmental stimuli or stress conditions, HSPs undergo phosphorylation and/or dephosphorylation. Interestingly, a negative correlation in the level of serine-phosphorylated HSP27 is observed in advanced HCC stages, whereas a positive correlation is found with the total HSP27 level under similar conditions (97). Regarding the signalling pathways involved, HSP90 is defined to have a role in IL-6/STAT-3 signalling (98). On the other hand, studies in other organs indicate that the functions of HSP72 have a positive role in modulating the gene expression controlled by NF-κB in sepsis (99).

However, whether other HSPs resemble the roles of HSP72 in interacting with the NF-κB pathway remains to be defined in human liver deficiency.

**Wnt pathway**

The Wnt-signalling pathway is another major signalling pathway related to HCC incidence and development (100). This signalling cascade is important for cell fate determination during embryonic development as well as for maintenance of tissue homeostasis. More importantly, it has been linked to HCC arising from several aetiological factors, such as HBV/HCV infections or alcoholic liver cirrhosis (101). Apart from livers, Wnt signalling has been implicated in other cancers, such as gastrointestinal and colorectal cancers (102, 103). In addition to Wnt, frizzled-7 and β-catenin are two candidates that participate in the Wnt-signalling pathway (104). Up-regulation of frizzled-7, accompanied by dephosphorylation of β-catenin, is frequently exhibited in HCC tissues (105, 106).

**MAPK pathway**

Many biological processes, including cell adhesion mechanisms, follow the MAPK pathway (107–109). This signalling pathway is highly involved in cell proliferation, cell differentiation and cell survival. In hepatic chronic inflammation, hepatitis viruses are known to act directly on different component proteins along the MAPK signalling pathways (110). For instance, HCV virtually has the ability to influence the extracellular signal-regulated kinase (ERK) pathway within the hepatocytes (110). In HCV-associated HCC, the MAPK/ERK pathway is activated, having a positive role in promoting HCC proliferation (111). As such, blocking these pathways with specific inhibitors is used as a means to map the pathways involved in these processes. Recently, the Sprouty-related protein with an Ena-/vasodilator-stimulated phosphoprotein homology-1 domain (Spred) was implicated as physiological inhibitor of the ERK pathway. Its...
expression level is inversely correlated with the incidence of tumour invasion and metastasis. It is noted that forced expression of this protein, which inhibits the ERK pathway, can successfully reverse the proliferation activities of HCC cells (112) through a reduction in the secretion of matrix metalloproteinase-9 (MMP-9) and MMP-2. These studies unequivocally reveal the role of the MEK–ERK pathway in HCC tumorigenesis. By assembling all this information, it is believed that phosphorylation and dephosphorylation of the pathway components can master the HCC process and promote tumour pathogenesis.

Growth factors/cytokines and HCC

Groups of growth factors are known to take part in HCC progression. Studies have uncovered the roles of vascular endothelial growth factor (VEGF) (113) and fibroblast growth factor (FGF) (114) in HCC development. On the other hand, cytokines, in combination with other growth factors, also share an essential role in modulating the HCC progression and development. Tumour necrosis factor α (TNF-α) is found to have dual roles in liver injury (115). Despite all those studies, no growth factors or cytokines have been discovered using the proteomics approach. However, emerging results relating to the differential expression of cytokines in different liver clinical situations have been identified using the genomics methodology. Strategies using function-blocking antibodies or specific inhibitors are used to verify their roles in HCC. The use of anti-transforming growth factor–β (TGF–β) antibodies demonstrated the suppression of HCC, illustrating the harmful role of excess TGF–β (116). Also, epidermal growth factor receptor (EGFR) inhibitors can prevent HCC development in the rat liver (117).

Other pathways

In addition to the mechanism mentioned above, other pathways also exist that take part in regulating the molecular dynamics of HCC. Cellular transport is an important aspect to ensure the successful translocation of targets to its destined locations. During the course of HCC progression, this event is usually hampered. MAL2, an integral protein necessary for delivering glycosylphosphatidylinositol-anchored protein, failed to carry out its task in the hepatoma cells (118). In addition, the classical physiological transporters, such as annexins and transferrin receptors, are also involved in HCC development (42, 119). Liver tumour development also affects other physiological processes, such as apoptosis (120), alcohol metabolism (121) and ubiquitin protein metabolism (122).

Concluding remarks and future perspectives

Hepatocellular carcinoma is a heterogeneous cancer with no promising treatment. Proteomics is used to identify panels of biomarkers for diagnosing HCC. Using protein biomarkers, the mechanism of the development and growth of tumour cells in livers can be elucidated. Recent clinical efforts to modulate hepatocarcinogenesis have stressed on the utilization of pathway inhibitors, such as the inhibitor against the MEK–ERK pathway that has anticancer properties (123), in this area, hoping to provide a new insight into designing a more effective treatment option for these detrimental diseases. More importantly, with the belief that phosphorylation and dephosphorylation of the MAPK pathway components can master the HCC process and promote tumour pathogenesis, the recently developed phosphoproteome technology searching for phospho-biomarkers may provide an area of research in the future. By combining this information, biomarker identification in the manipulation of the HCC-related signalling pathway is an alternate approach in the line of diagnosis and prognosis of this disease. This knowledge not only advances the understanding of the malignancy, but at the same time can provide clues on the therapeutic treatment and prognosis of liver pathogenesis. New efforts are necessary to analyse systematically the cancer signalling phosphoproteomes in response to growth/angiogenic factors and stress conditions in the tumour microenvironment.

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