5 Genomic Profiling of Human Hepatocellular Carcinoma

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ABSTRACT

Numerous studies of human gene function have been launched since the sequencing of the human genome. Global molecular profiling studies of hepatocellular carcinoma (HCC) are providing a comprehensive view of the expression changes that occur during the carcinogenic process and are uncovering promising biomarkers with clinical potential. In this chapter, an overview of recent gene expression profiling of human HCC is provided along with a summation of the mechanistic, diagnostic, and prognostic significance of these findings. Emerging concepts associated with these studies are also addressed and biomarkers present in serum are highlighted. Current

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profiling studies, conducted on multiple array platforms, are powerful tools which have provided useful clues to begin to unravel the mechanisms of HCC biology and improve clinical outcome.

Key Words: Hepatocellular carcinoma; molecular marker; gene expression profiling; microarray; liver disease

1. HEPATOCELLULAR CARCINOMA: CLINICAL CONCERNS

The wide heterogeneity of HCC and the complexity of its diagnostic and prognostic assessment (dependent on tumor grade/residual liver function) have interfered with clinical recommendations and progress. Despite many studies of HCC, the specific changes associated with its development remain ill defined and there is no clear consensus on which of the many different staging systems introduced around the world is best *(1–4)*. Although individuals at high risk for HCC development are routinely screened by ultrasonography and serum α-fetoprotein (AFP), most patients are diagnosed at advanced disease stages. AFP evaluation, however, can be non-specific, varies significantly between ethnic groups, and is only observed in a HCC subgroup with small tumors *(5)*. Although several additional serum proteins have been suggested to improve HCC diagnosis, they lack sensitivity and specificity and await confirmatory studies or development of quantitative methods to evaluate their utility *(6, 7)*. It is possible that a single marker may not be sufficient to diagnose HCC and as such, it may be important to test combinations of markers to improve diagnostic performance. HCC diagnosis with the AFP marker, therefore, remains the gold standard and improvement of the current screening system is an imperative goal. Liver function impairment and the expression of multidrug resistance genes render HCC treatment especially difficult *(8)*. Since most HCC patients are diagnosed at an advanced stage, they are often excluded from potentially curative therapies such as resection and liver transplantation. Eligibility for resection (relatively good liver function and small tumors) or transplantation (Milan criteria/limited donor livers/long waiting list) is also quite slim and post-surgical survival is complicated by a predominant occurrence of tumor recurrence/metastasis *(9–15)*. Methods to improve survival include percutaneous ethanol injection, radiofrequency ablation, and transarterial chemoembolization (TACE) *(16)*.

The current status of HCC emphasizes the importance of understanding the underlying biology of this disease and the development of new screening and treatment stratification programs to refine diagnosis and improve patient outcome. Relevant biomarkers to assist HCC diagnosis and prognosis are particularly essential at early HCC stages and can be used as

novel therapeutic agents. The identification of such biomarkers in a high-throughput fashion is now possible through the advent of global molecular profiling.

2. GENE EXPRESSION PROFILING: CURRENT TECHNOLOGIES

The gene expression profile of a particular cell type or tissue has been analyzed by using multiple technologies including differential screening of cDNA libraries, subtractive cDNA hybridization, differential display of RNA, and serial analysis of gene expression (SAGE). More recently, global expression profiling studies have been conducted using platforms consisting of genes (cDNA/OLIGO microarrays), noncoding RNA, proteins (proteome arrays), tissues (tissue microarray), and genetic aberrations (arrayCGH/methylation) *(17)*. Although previous methodologies to study HCC have advanced the field, gene expression profiling of clinical samples from HCC patients and HCC-related cell lines has enriched the breadth of HCC knowledge and has allowed researchers to begin to tackle some of the key disease-related concepts that still remain.

2.1. Microarray Platforms

Microarrays provide genomic information and insight into biological processes on a genome-wide scale. Their miniaturized ordered arrangement of targets (nucleic acids/proteins/tissues) located at defined positions on a solid support (platform) enables high-throughput parallel analysis of many targets by specific hybridization. The composition of an array platform can be global (an entire genome on a slide) or specific (pathways, cell/tissue type) and allows for the characterization of a transcriptome/proteome/genome. A brief overview of widely used array platforms is provided below.

2.1.1. Expression Arrays (cDNA/OLIGO/Noncoding RNA)

The cDNA microarray reports differences in gene expression levels between samples and functions on the basis of specific and high-affinity molecular recognition between complementary cDNA strands (PCR-derived cDNA or 20–60mer OLIGO fragments) representing exonic regions of the genome *(18)*. The regulation of mRNAs can be analyzed using microRNA (miRNA) arrays, which globally interrogate the expression of small endogenous (21–35 nt) noncoding RNAs. Platforms that detect mature and precursor forms of >500 miRNAs are now commercially available *(19–21)*.

2.1.2. Protein Arrays (Proteome/Tissue)

Although mRNAs are transcribed, they may not be translated and thus mRNA copy number may not reflect the number of functional protein molecules in a cell. Thus, proteome arrays may provide a better view to understand gene function. Protein function or protein detecting arrays involve immobilization of antibody probes to detect antigens in a sample, or vice versa. These arrays can be used to quantify proteins, determine post-translational modifications, and correlate proteins with disease advancement or with certain treatments/environments *(22)*. Tissue microarrays (TMA) allow tissue-based profiling using small cylinders of formalin-fixed tissues arrayed in a single paraffin block *(23)*. Protein arrays are limited by the protein concentration range required for direct detection within a given sample and current instrumentation allows for only a fraction of the proteome to be examined. The measurement of low-abundance targets also remains a challenge, but high-affinity probes, such as SELEX (systematic evolution of ligands by exponential enrichment) aptamers, can help to resolve this problem *(24, 25)*.

2.1.3. Genomic Arrays (CGH/Methylation)

Array comparative genomic hybridization (aCGH) using the BAC-based (bacterial artificial chromosome) and the more recent oligonucleotide-based CGH enables high-resolution multi-loci mapping of small genomic regions with copy number changes, such as amplification or deletion *(26, 27)*. BAC aCGH is limited by costly, time-consuming, low-yield clone production and noisy data due to non-specific hybridization of repetitive sequences. Oligonucleotide aCGH allows for flexibility in probe design, greater genomic coverage, and higher resolution (~50 kB). New tiling BAC arrays, however (where each BAC overlaps with its contiguous BAC), can increase resolution, signal intensity, and more accurately define the boundaries of genomic aberrations, but require a high concentration of high-quality BAC DNA for good array performance *(28, 29)*. Recently, a few CGH array studies have been followed by bisulfate DNA sequencing or methylation-specific PCR to identify HCC-related epigenetic changes.

2.2. Microarray Analysis

Methodologies for microarray analysis can be either unsupervised or supervised *(30–32)*. Unsupervised methods attempt to characterize the components of a data set without a priori input or knowledge of a training set. Internal structures or relationships in data sets are found by feature determination which groups genes with interesting properties (principal component analysis), cluster determination which groups genes or samples with similar patterns of gene expression (nearest-neighbor clustering,

self-organizing maps, k-means clustering, and one- and two-dimensional hierarchical clustering), and network determination which graphs gene–gene or gene–phenotype interactions (Boolean networks, Bayesian networks, and relevance networks). On the other hand, supervised methods are used to determine genes that fit a predetermined pattern. This technique finds genes with expression levels that are significantly different between groups of samples (e.g., cancer classification) and can be used to find genes that accurately predict a characteristic of that sample (e.g., survival or metastasis). The significance found by supervised methods has been evaluated using parametric, non-parametric, and analysis of variance procedures which involve permutations, random partitioning of the studied data set, and false discovery limits. These methods are employed to assess the validity of signatures associated with a tested feature and to rule out the identification of a signature by random chance. Several criteria exist for determining differential expression, including absolute or ratio of expression levels across samples and subtractive degree of change between groups. These methods include the nearest-neighbor approach, decision trees, neural networks, and support vector machines. A gold standard has been proposed for analysis of array studies which involves the use of a training data set to initially identify a signature, a test data set to assess its predictive/classification capacity, and an independent set for validation studies.

3. HCC MICROARRAY STUDIES: EMERGING CONCEPTS

Microarray studies have provided vast amounts of information concerning the genes, proteins, and genomic changes that occur in HCC-related disease. These investigations have revealed changes that occur across a spectrum of cirrhosis, HCC tumors, HCC subtypes, epigenetic alterations, and progressive phenotypes (metastasis/recurrence). A summary of these signatures, affected pathways, and diagnostic/prognostic markers is provided in Table 1. An overview of these studies along with a synopsis of emerging perspectives gleaned from these analyses is provided in this section.

3.1. Diagnostic HCC Signatures

3.1.1. Chronic Liver Disease Signatures

HCC develops largely in a previously diseased liver, contributed by chronic liver disease (CLD). CLD has been attributed to hepatitis viral attack, genetic/metabolic disorders, alcohol abuse, and/or environmental influences *(13, 33)*. The HCC population is, therefore, quite heterogeneous, since the tumor and CLD can be at different evolutionary stages at diagnosis, each with different therapeutic perspectives and survival probabilities.

Table 1
HCC Microarray Signatures[a]

Platform	*Prediction signatures*	*Sample size*	*Study design*				*Affected pathways*	*Ref*
			Training set	*Testing set*	*Independent set*	*Validation method*		
Genome	6 genes	20 cases	Yes	No	No	No	No	*(78)*
	13 regions	104 HCCs; 76 non-HCCs	Yes	No	No	No	No	*(230)*
	4 gain regions; 7 lost regions	19 HCV– HCCs	Yes	No	No	No	No	*(77)*
	673 clones	44 cases; 5 HCC cell lines	Yes	No	No	No	No	*(76)*
	2 prognostic regions	87 cases	Yes	No	No	FISH	No	*(81)*
	8 gain regions; 9 LOH regions	36 cases	Yes	No	No	No	No	*(231)*
	7 genes	88 cases	Yes	No	No	qRT-PCR; ISH	No	*(109)*
	3 regions	63 HCCs; 4 HCC cell lines	Yes	No	No	qRT-PCR; WB; IH	Etiology specific	*(96)*

Table 1
(Continued)

Platform	*Prediction signatures*	*Sample size*	*Study design*				*Affected pathways*	*Ref*
			Training set	*Testing set*	*Independent set*	*Validation method*		
cDNA	HCC patho/ virological genes	20 cases	Yes	No	No	No	Wide range	*(68)*
	2253 genes	4 cases	Yes	No	No	RT-PCR	Cell cycle; Wnt signaling	*(40)*
	9 HCC genes; 22 differentiation genes	10 cases	Yes	No	No	No	No	*(63)*
	HCC-specific genes	102 HCCs; 74 non-HCCs; 7 BLTs; 10 MCs; 10 HCC cell lines	Yes	No	No	No	P53; Vascular invasion	*(113)*
	HCV-cirrhosis genes	6 HCV– HCCs; 4 AIHs; 8 NLs	Yes	No	No	RT-PCR	Wide range	*(232)*

(*Continued*)

Table 1
(Continued)

			Study design					
Platform	*Prediction signatures*	*Sample size*	*Training set*	*Testing set*	*Independent set*	*Validation method*	*Affected pathways*	*Ref*
	HCC subgroup genes	18 HCCs and 1 HB cell line	Yes	No	No	No	AFP	*(101)*
	HCC patho/ virological genes	15 cases	Yes	No	No	RT-PCR	Wide range	*(67)*
	90 clones	22 HCC foci	Yes	No	No	No	No	*(114)*
	83 genes	14 HBV–HCCs; 31 HCV–HCCs	Yes	No	No	No	No	*(45)*
	HCC-specific genes	8 cases	Yes	No	No	RT-PCR	No	*(57)*
	50 genes	20 HCV–HCC cases	Yes	Yes	Yes	RT-PCR; NB	No	*(58)*
	30 genes	30 cases	Yes	Yes	Yes	RT-PCR; IH; WB	No	*(122)*
	83 genes	17 WT-p53 cases; 5 MT-p53 cases	Yes	No	No	RT-PCR	P53	*(66)*

Table 1
(Continued)

Platform	*Prediction signatures*	*Sample size*	*Study design*				*Affected pathways*	*Ref*
			Training set	*Testing set*	*Independent set*	*Validation method*		
	3 genes	33 cases	Yes	Yes	Yes	No	Wide range	*(135, 136)*
	68 genes	37 cases	Yes	Yes	Yes	RT-PCR	Wide range	*(61)*
	59 genes	10 cases	Yes	Yes	No	No	No	*(62)*
	220 genes	20 HCCs; 17 non-HCCs; 31 NLs	Yes	Yes	Yes	No	No	*(55)*
	89 HBV–HCC /9 HCV–HCC genes	14 HBV–HCCs; 31 HCV–HCCs	Yes	No	No	RT-PCR	No	*(93)*
	30 genes	14 HCCs; 7 HBVs; 11 HCVs; 3 HHCs; 5 WDs; 16 PBCs; 10 ALDs; 7 AIHs	Yes	Yes	Yes	No	No	*(90)*
	20 genes	100 cases	Yes	Yes	No	No	No	*(129)*

(*Continued*)

Table 1
(Continued)

Platform	Prediction signatures	Sample size	*Study design* Training set	Testing set	Independent set	Validation method	Affected pathways	Ref
	406 genes	91 HCCs; 60 non-HCCs; 18 NLs	Yes	Yes	No	No	Cell cycle; Apoptosis	*(119)*
	668 genes	7 Solitary large HCCs; 15 Nodular HCCs	Yes	No	No	RT-PCR; WB; IH	No	*(233, 234)*
	129 genes	12 LC nodules; 5 HCCs	Yes	Yes	No	No	No	*(91)*
	HCC subtype genes	43 HCCs; 3 HCC cell lines	Yes	No	No	RT-PCR; NB	Apoptosis; Immune response	*(100)*
	44 genes	33 HCCs; 23 non-HCCs	Yes	Yes	Yes	RT-PCR	No	*(59)*
	4 genes	33 cases	Yes	Yes	Yes	RT-PCR; IF	Immune response	*(235)*
	240 genes	50 hepatocellular nodules	Yes	Yes	No	No	No	*(105)*
	Chromosome-specific HCC genes	31 HCCs; 19 non-HCCs	Yes	No	No	qRT-PCR	No	*(236)*

Table 1
(Continued)

Platform	*Prediction signatures*	*Sample size*	*Study design*				*Affected pathways*	*Ref*
			Training set	*Testing set*	*Independent set*	*Validation method*		
	38 genes	20 cases	Yes	No	No	No	No	*(51)*
	Liver-fibrosis genes	3 non-HCV cases; 19 HCV cases	Yes	No	No	RT-PCR	No	*(92)*
	25 genes	13 LCs; 23 non-tumor LCs near HCC; 19 HCCs	Yes	No	Yes	qRT-PCR	No	*(44)*
	63 genes	6 HCAs; 8 well-differentiated HCCs	Yes	Yes	Yes	qRT-PCR; IH	No	*(102)*
	HBV–HCC genes	15 HCCs; 5 non-HCCs	Yes	Yes	No	qRT-PCR	c-myc	*(237)*
	HCC subgroup genes	61 human HCCs; 39 mouse HCCs; Rat fetal hepatoblasts; Rat adult hepatocytes	Yes	Yes	No	No	AP-1 activation	*(142)*

(*Continued*)

Table 1
(Continued)

			Study design					
Platform	*Prediction signatures*	*Sample size*	*Training set*	*Testing set*	*Independent set*	*Validation method*	*Affected pathways*	*Ref*
	36 genes	40 cases (28 SNs, 12 MNs)	Yes	Yes	No	No	No	*(60)*
	31 genes	24 cases	Yes	No	No	RT-PCR	Cell cycle; Immune response	*(103)*
	14 genes	18 cases	Yes	Yes	Yes	qRT-PCR	No	*(130)*
	35 genes	35 cases	Yes	No	No	qRT-PCR; WB	No	*(238)*
	46 genes	35 cases	Yes	No	No	RT-PCR	Cell adhesion	*(134)*
	123 genes	2 $HBV^{-}HCV^{-}$ HCCs; 2 HBV^{-}HCCs; 2 HCV^{-}HCCs; 6 non-HCCs	Yes	No	No	No	Cell cycle; Cell adhesion	*(118)*
	40 genes	1 NIN HCC; 3 dysplastic nodules; 3 HCCs	Yes	No	No	RT-PCR	No	*(104)*

Table 1
(Continued)

Platform	*Prediction signatures*	*Sample size*	*Study design*				*Affected pathways*	*Ref*
			Training set	*Testing set*	*Independent set*	*Validation method*		
	17 genes	115 cases	Yes	Yes	Yes	qRT-PCR; IH	Immune response	*(138)*
	217 genes	40 cases	Yes	No	No	NB; IH	No	*(115)*
	Angiogenesis soluble factors	38 HCV–HCCs; 52 HCV–LCs; 6 NLs	Yes	No	No	qRT-PCR	Angiogenesis	*(50)*
	5 genes	218 cases	Yes	Yes	Yes	qRT-PCR; ELISA	No	*(54)*
	57 genes	48 cases	Yes	Yes	Yes	qRT-PCR	Wide range	*(137)*
	248 genes	40 cases	Yes	No	No	RT-PCR; NB; IH	No	*(239)*
	2 genes	40 cases	Yes	Yes	Yes	qRT-PCR	Wnt	*(144)*
microRNA	8 miRNAs	24 HCCs; 22 non-HCCs	Yes	No	No	NB	No	*(89)*
	35 miRNAs	17 HCCs; 21 LCs	Yes	No	No	qRT-PCR; NB	No	*(95)*
	15 miRNAs	3 cases	Yes	No	No	qRT-PCR; NB	No	*(240)*
	35 miRNAs	17 HCCs; 21 LCs	Yes	No	Yes	qRT-PCR; NB	No	*(95)*

(*Continued*)

Table 1 (Continued)

Platform	*Prediction signatures*	*Sample size*	*Study design*				*Affected pathways*	*Ref*
			Training set	*Testing set*	*Independent set*	*Validation method*		
	40 miRNAs	10 cases (without virus)	Yes	No	No	NB	No	*(241)*
	20 miRNAs	131 cases	Yes	Yes	Yes	qRT-PCR	No	*(123)*
	22 miRNAs	19 cases	Yes	No	No	qRT-PCR	No	*(86)*
Proteome	32 proteins	30 HCCs; 15 NLs	Yes	No	Yes	WB	No	*(73)*
	250 features	20 CLDs; 38 HCCs	Yes	Yes	No	No	No	*(97)*
	90 proteins	67 cases; 12 NLs	Yes	No	Yes	WB; IH	Cell growth; angio-genesis	*(71)*
	4 peptides	34 HCC serums; 39 F1/F2 fibrosis serums; 44 F4 fibrosis serums	Yes	No	No	No	No	*(99)*
	11 spots	20 cases	Yes	No	No	WB	No	*(69)*
	15 signals	25 HCCs; 23 HCC margins; 28 NLs;	Yes	No	No	IH	No	*(72)*

Table 1
(Continued)

Platform	*Prediction signatures*	*Sample size*	*Study design*				*Affected pathways*	*Ref*
			Training set	*Testing set*	*Independent set*	*Validation method*		
	12 peaks	26 SNs; 45 MNs; 6 HCCs	Yes	No	No	No	No	*(41)*
	6 proteins	28 HCC serum; 18 normal serum	Yes	No	Yes	WB	No	*(242)*
	11 peaks	41 HCC serums; 51 HCV–LC serums	Yes	No	No	No	No	*(98)*
Others								
Genome/regional expression bias	2 regions	39 HCCs	Yes	No	No	No	No	*(82)*

(*Continued*)

Table 1
(Continued)

			Study design					
Platform	*Prediction signatures*	*Sample size*	*Training set*	*Testing set*	*Independent set*	*Validation method*	*Affected pathways*	*Ref*
Genome/ cDNA array	Correlation between genomic copy and cDNA level	41 HCCs; 12 HCC cell lines	Yes	No	No	PCR	No	*(79)*
Genome/ cDNA array	31 positive-correlated genes	20 cell lines	Yes	No	No	qRT-PCR	No	*(83)*
Proteome/ cDNA array	93/125 correlated proteins	14 cases	Yes	No	No	No	No	*(243)*

Note
[a] The papers cited in this table are microarray data performed on clinical HCC samples.

AIH—Autoimmune hepatitis; ALD—alcoholic liver disease; BLT—benign liver tumor; CLD—chronic liver disease; HB—hepatoblastoma; HCA—hepatocellular adenocarcinoma; HCC—hepatocellular carcinoma; HHC—hemochromatosis; LC—liver cirrhosis; LOH—loss of heterozygosity; MC—metastatic cancer; MN—multinodular; MT—mutant-type; NIN—nodular in nodular; NL—non-disease liver; PBC—primary biliary cirrhosis; SN—single nodular; WD—Wilson's disease; WT—wild type.

ELISA—Enzyme-linked immunosorbent assay; IH—immunohistochemistry; ISH—in situ hybridization; NB—northern blot; RT-PCR—reverse transcription polymerase chain reaction; qRT-PCR—quantitative RT-PCR; WB—: Western blot.

Several gene expression profiling studies have focused on CLD etiologies (mainly of hepatitis B and/or C viral infection) in order to identify diagnostic markers, particularly for early detection. cDNA arrays have shown that genes associated with the TH1 immune response (including lymphocyte/monocyte activation), fibrosis, extracellular matrix remodeling, cell–cell interactions, proliferation, cell growth regulation, and apoptosis are upregulated in HCV–CLD *(34–36)*. Candidate genes ($n = 260$) involved in signal transduction pathways, cell cycle control, metastasis, transcriptional regulation, immune response, and metabolism were aberrantly expressed under HBx induction by cDNA array *(37)*. In our laboratory, we have shown that primary hepatocytes expressing HBx have altered expression of several cellular oncogenes and tumor-suppressor genes *(38)*. Oncogenes, cell cycle regulators, intracellular transducers, stress response genes, apoptosis-related genes, and transcription factors were also shown to be upregulated in response to HBV infection, while growth factors were downregulated *(39)*. Several of these HBV-altered genes were correlated to regions with amplification (1q, 8q, 13q) or loss of heterozygosity (4q, 8p, 16q, 17p) *(40)*. In addition, global proteomic profiling has shown that cirrhotic nodules in a HBV background contain signatures associated with clonal expansion *(41)*.

The differentially expressed genes altered by HBV and HCV infection have also been analyzed using microarrays. Differential gene expression was shown by cDNA array between chronic HBV and HCV hepatic lesions, with HBV-affecting genes related to inflammation while HCV-affected genes related to the anti-inflammatory process *(42)*. However, only a slight difference between HBV and HCV host cell infection was found in another cDNA array, but the authors noted that the differentially expressed genes were clearly regulated in a reciprocal manner *(43)*. Other cDNA studies have shown that lectin and cytochrome p450 can distinguish viral cirrhosis subtypes *(44)*. In an OLIGO-based study, 83 genes were found to differ between HBV and HCV–HCC, including those related to signal transduction, metastasis, and immune response *(45)*. Another OLIGO array study revealed 176 genes that were altered upon HBV or HCV viral infection, including the interferon-inducible gene IFI27 *(46)*. IFI27 was also shown to be highly upregulated in HCV–HCC in an OLIGO array-based study in our laboratory in which human hepatocytes were infected with HBV- or HCV-related genes *(47)*. OLIGO arrays have also shown that an HCV-specific gene (NS5A) can modify pathways associated with cell motility and adhesion, lipid transport and metabolism, calcium homeostasis and regulate the immune response through NF-kB signaling *(48, 49)*. The strongest effects were a downregulation of an adenylate synthetase (OAS-69) and an upregulation of IL8 which both affect IFN anti-viral activity. In a proteomic array study, angiogenic factors, including VEGF, were upregulated in HCV–HCC tissues *(50)*.

Taken together, these observations suggest that a high degree of changes take place in CLD tissues. The identification of these premalignant changes may be useful to classify patients with CLD groups or those patients at risk for developing HCC. In addition, these notable changes involved in CLD may be useful for early detection and thus provide a window of opportunity to intervene with an effective therapy. These studies have also demonstrated that some genes are consistently altered in preneoplastic conditions and HCC, highlighting early changes that may also play a role in disease progression. Many of these studies, however, involve relatively small cohorts, identify relatively large signatures/classifiers, do not provide sufficient follow-up data to confirm patient outcome, or are not validated in independent cohorts. Therefore, large prospective studies and/or meta-analysis of existing data sets will be needed to validate the potential clinical use of these CLD-related markers as diagnostic tools.

3.1.2. Tumor Biomarkers (Tumor vs Non-tumor)

Microarray studies have also enhanced our understanding of how the HCC process alters the regulatory network of genes and proteins in a way that differs from the respective normal tissue or disease-free samples. For example, cDNA analysis of HCC vs normal samples has found 38 differentially expressed genes while HBV-related cell lines revealed signatures (356 genes) composed of upregulated ribosomal-related genes *(51, 52)*. TIPUH1, a regulator of transcription and RNA processing of growth control genes, has also been shown to be upregulated in HCC by cDNA array *(53)*. In our laboratory, we have shown that five genes (GPC-3, PEG10, MDK, SERPINI1, and QP-C) are elevated in HCC samples, even in those with low AFP status compared to normal tissue *(54)*. A cDNA array of non-HBV/HCV-infected HCC vs normal tissues revealed 61 differentially expressed genes *(55)*. A number of studies have also found alterations in genes involved in protein synthesis, growth factors, oncogenesis, stress, inflammation, cell proliferation, transcription, protein degradation, p53, Wnt/β-catenin, metabolism, and tumorigenesis pathways in HCC *(40, 56–62)*. Similar studies have shown that activators of neutrophils, antiapoptotic genes, interferon response genes, and proteins related to cell differentiation or development are differentially expressed in HCV–HCC *(63)*. Integrin and Akt/NF-kB signaling were also upregulated in HCC along with a serum biomarker (CSTB) using cDNA arrays *(64, 65)*. OLIGO arrays have shown that p53-related genes ($n = 83$) are affected by HCV infection and alter immune response, transcription, transport, signal transduction, and metabolism in tumors *(66)*. Several of these pathways, along with growth factor alterations, were found in cDNA arrays comparing HBV- or HCV-positive tumor with non-tumor

tissue *(67)*. A clear distinction was found between HBV and HCV samples, where HBV-affected genes were involved in apoptosis, p53, and the G1/S transition while HCV-affected genes were more heterogeneous. In a separate cDNA array study, upregulation of mitosis-promoting genes was observed in the majority of HBV or HCV tumors vs non-tumors while differentially expressed genes between HBV and HCV tumors encoded enzymes that metabolize carcinogens and/or anticancer agents associated with malignant/invasive phenotype, apoptosis, or immune regulation *(68)*.

Proteomic and TMA arrays have also been used to address the differences that occur following tumor formation. A proteomic analysis of human HCV-related HCC found alterations in glycolysis enzymes, mitochondrial β-oxidation pathways, and cytoskeletal proteins when compared to non-tumor tissue *(69)*. Other HCC-related protein classifiers include proteins involved in heat shock response, glycolysis, fatty acid transport and trafficking, amino acid metabolism, cell cycle regulation and cell stress, and metabolism-related enzymes *(70–72)*. Other upregulated genes in HCC include insulin growth factor-II, metalloproteases, signal transducers and activators of transcription (STAT), suppressors of cytokine signaling, and cyclin D1 while collagens and SMAD pathways were downregulated *(73)*. A TMA study of HCC/non-tumor comparisons found HCC-specific expression of the transcription repressor zinc fingers and homeoboxes 2 (ZHX2) protein expression which correlated with differentiation stage *(74)*.

Multiple studies have aimed to determine HCC-related regions of genetic gain or loss. Most studies have found similar regions of gain (1p, 4q, 8p, 13q, 16q, and 17p) and loss (1q, 6p, 8q) in HCC *(75–77)*. In addition, a study of 120 HCC samples found LOH at 6q and 9p in small, well-differentiated tumors *(75)*. A comparison of tumor vs non-tumor HCC samples using BAC aCGH included frequent DNA copy number gains of 20q and found that high Jab1 levels correlated with chromosome 8q gain in HCC *(76)*. A study of HCV-associated HCC revealed that increases of DNA copy number were frequent at 10p while decreases were frequent at 10q *(77)*. These authors found increases in copy numbers of the LAMC2, TGFB2, and AKT3 genes (located on 1q) and decreases in copy numbers of FGR/SRC2 and CYLD (located on 1p and 16q, respectively) in tumors. In a study of 20 HCC cases, oncogenes were amplified in 1q, 8p, and 11q regions while loss occurred at 13q and 4q *(78)*. In a study of HBV-infected HCC, gains on 1q, 6p, 8q, 9p were observed while losses in 1p, 16q, and 19p occurred in most patients *(79)*. Midorikawa et al. showed a frequent gain of 1q, 8q, 12q, 17q, and 20q as well as a loss of 4q, 8p, 13q, and 17p in HCC *(80)*. Gains in regions encoding MET, c-myc, and FGF4 were also found in a CGH study of HCC while a separate study identified narrow regions of frequent amplification on chromosome 1p, frequent deletion on 17q, and alterations in 7q21 encoding paternally expressed 10 (PEG10) *(81–83)*.

miRNAs have recently been utilized as potential HCC diagnostic markers. Expression profiling studies have defined the liver-specific miR-122 to be highly downregulated in HCC tumors and cell lines *(84)*. miRNA array studies have also demonstrated that miR-21 can contribute to HCC growth and spread by modulating PTEN *(85)*. In other miRNA-based studies, mir-224, a 16-miRNA set, and a novel mRNA-like noncoding RNA named highly upregulated in liver cancer (HULC) were found to be significantly upregulated in HCC *(86–88)*. In another study comparing HCC samples and adjacent non-tumor, eight miRNAs were shown to be significantly altered, five of which were downregulated in HCC and could predict HCC with 97% accuracy *(89)*.

3.1.3. Tumor Biomarkers (Tumor vs Cirrhosis)

Array-based comparisons have also been made between early neoplastic stages (fibrosis/cirrhosis) and HCC. A study of 59 preneoplastic CLDs (hepatitis, autoimmune hepatitis, primary biliary cirrhosis, etc.) conducted in our laboratory found genes associated with high or low risk of HCC development *(90)*. This 273-gene signature was validated in three independent cohorts and included 12 secretory genes in the top gene set. In separate cDNA array-based studies, 25 cirrhosis-specific genes were identified that were related to inflammatory status of adjacent HCC tissue and 129 genes were altered in HCC compared to liver cirrhosis samples *(44, 91)*. In an OLIGO array-based study of fibrosis, carbohydrate metabolism genes were elevated in HCC patients when compared to cases with F3-4 fibrosis *(92)*. In a comparison of HCC with CLD (either HBV or HCV positive) or HCC without CLD in an OLIGO array, genes involved in transcription, metabolism, and cell growth were differentially expressed *(93)*. An RT-based study of cirrhosis vs HCV–HCC showed that twelve genes were significantly altered (including GPC3, TERT, survivin, XLKD1, and CDH1) *(94)*. MiRNA platforms have also demonstrated that 35 miRNAs including let7 and miR-181 family members differ between HCC and cirrhosis *(95)*. aCGH of 63 HCCs found etiology-dependent copy number gains, including 8q24 and MYC overexpression in viral and alcohol-related HCCs *(96)*. The use of comprehensive proteomic profiling of sera to differentiate HCC from CLD found 250 significantly different proteins, while an 11-peak SELDI profile or 4-peptide panel could distinguish HCC from HCV-related cirrhosis and was an independent predictor of HCC *(97–99)*.

3.1.4. Tumor Biomarkers (Tumor Subtype Signatures)

Several HCC array studies have also compared HCC tumors to identify subtypes or to compare various tumor stages or nodular status to understand the changes that occur between early and late tumorigenesis. In a cDNA study of HCC and HCC cell lines, two subgroups of HCC were

identified that were related to either IFN-associated inflammation or apoptosis, while another cDNA study composed of 19 HCC cell lines found 2 subtypes that were correlated with AFP expression *(100, 101)*. In a comparison of multinodular and solitary HCC, cDNA arrays revealed 230 genes that were specific to multinodular recurrence, while only 36 were commonly expressed *(60)*. A separate cDNA study of HCCs from 10 patients found several genes related to histological subtype *(62)*. In an OLIGO study of well-differentiated HCC vs hepatocellular ademonas, 63 genes were found to be differentially expressed, demonstrating molecular differences despite similarities in morphology *(102)*. Another OLIGO study identified 31 genes that differed between early and advanced HCV–HCCs *(103)*. In other OLIGO-based studies analyzing nodule-in-nodule HCC, dysplastic nodules, and HCCs, the authors found 40 genes involved in the transition from dysplasia to early-stage tumors and 240 genes that could accurately classify tumors according to histological grade *(104, 105)*.

3.1.5. Tumor Biomarkers (Epigenetic Signatures)

HCC development is thought to be a multistep process involving not only accumulation of genetic changes but also epigenetic changes, such as methylation, which can reversibly alter regulatory genes. A few studies have begun to address the epigenetic changes that occur in HCC. In a cDNA/bisulfite PCR study, the demethylating agent 5-Aza-dC was used to identify hepatocyte growth factor (HAI-2/PB) as a frequent hypermethylated gene in HCC *(106)*. In another cDNA array and bisulfite PCR study, insulin-like growth factor-binding protein was found to be hypermethylated and down-regulated in HCC *(107)*. An OLIGO-based analysis of human HCC cell lines showed that treatment with 5-Aza-dC resulted in a decrease of the tissue factor pathway inhibitor TFPI-2 *(108)*. In addition, Pang et al. found a loss of an unmethylated 6q allele in HCC encoding a putative tumor-suppressor gene *(109)*. However, in a study of 60 primary HCCs using aCGH and methylation-specific PCR, a causal relationship was not observed between the methylation status of nine CpG islands, including p16, COX2, and APC, and patient outcome *(110)*.

Thus, numerous array studies have shown that multiple tumor-specific alterations occur during hepatocarcinogenesis. A detailed exploration of these changes may offer new insight regarding HCC biology and provide avenues for diagnostic advances. Within platform types, however, marker sets are quite different from one another, despite a similarity in comparison groups which could be due to platform makeup, sample heterogeneity, differences in etiology or ethnicity among samples. In addition, many of these studies lack validation and are only drawn from a rather small data set, and therefore further studies will be needed to determine whether the identified changes can be widely useful for diagnostic or HCC classification purposes.

In sum, these studies clearly demonstrate that measurable changes occur during HCC development that may be useful for early detection.

3.2. Prognostic HCC Signatures

3.2.1. Metastasis/Survival/Recurrence Signatures in HCC Tumor or Non-tumor Tissues

Metastasis and recurrence are major factors affecting the outcome of patients with HCC. Understanding the mechanisms involved in the process of tumor invasion and metastasis is a major challenge. Biomarkers related to these processes may have clinical prognostic utility. Important questions related to metastasis involve initiation, the relationship between primary and metastatic tumors, and whether these metastatic changes are inherent to the cell or are acquired through time and/or environmental status. The current metastasis model suggests a multistage carcinogenic process initiated by rare genetic alterations in a single cell, followed by clonal selection and population expansion *(111)*. In HCC, however, such stepwise and specific progression-related genetic changes have not been illustrated *(3)*.

The transcriptome, proteome, and genome of metastatic HCC cells have been studied using array technology. Comprehensive cDNA analysis of HCV-related HCCs has identified 35 genes involved in portal vein invasion (PVI) including the inhibitor of DNA binding 2 (ID2), encoding a liver-rich dominant-negative helix–loop–helix protein which was validated by qRT-PCR, Western blot analyses, and in an independent set *(112)*. A 91-gene vascular invasion signature was also found in a separate cDNA study and 90 clones were correlated with intrahepatic metastasis in a study of 22 HCC foci *(113, 114)*. Another cDNA study of HCC found 217 genes associated with differentiation status and metastasis, including ANXA2 *(115)*. A cDNA array was also employed to profile gene expression patterns in two subtypes of HCC, solitary large HCC (SLHCC) and nodular HCC (NHCC), which differ significantly in metastatic incidence *(116)*. A significant decrease in RhoC expression in SLHCC compared to NHCC was strongly correlated with HCC metastasis, implicating RhoC as a potential prognosis marker and therapeutic target for HCC *(117)*. Another cDNA-based study found that HCC with high expression of ubiquitin-conjugating enzyme, Ube2c, displayed PVI and poor disease-free survival rates while 906 genes were found to differ between HCC and surrounding tissue, generating clusters (A and B) that were associated with patient survival *(118, 119)*. OLIGO array studies have also shown that MAPK pathway and angiogenesis factors such as VEGF and HGF are associated with HCV–HCC while 39 genes were significantly correlated with metastasis, including cortactin, a cortical actin-associated protein substrate of Src *(50, 120, 121)*. In our laboratory,

we have applied cDNA arrays to show that intrahepatic metastatic lesions are indistinguishable from their primary HCC, while primary metastasis-free HCC was distinct from primary HCC with metastasis *(122)*. These data indicate that primary HCC with metastatic potential is an inherent quality of the primary tumor rather than a capability acquired over time through mutation. The 153-HCC metastasis gene signature, whose lead gene was osteopontin (OPN), could accurately classify metastatic HCC. In our laboratory, we have also investigated whether certain miRNAs are associated with HCC metastasis *(123)*. We identified a unique 20-miRNA metastasis signature that could significantly predict ($p < 0.001$) primary HCC tissues with venous metastases from metastasis-free solitary tumors. A survival risk prediction analysis revealed that a majority of the metastasis-related miRNAs were associated with survival. Furthermore, the 20-miRNA tumor signature was validated in 110 additional cases as a significant independent predictor of survival ($p = 0.009$) and was significantly associated with survival and early-stage HCC. These 20 miRNAs may provide a simple profiling method to assist in identifying HCC patients who are likely to develop metastases/recurrence.

TMAs and aCGH have also been used to study HCC metastasis. The clinical significance of FGF3 overexpression was studied by TMA in 60 pairs of primary/metastatic HCCs and showed that overexpression of FGF3 was significantly associated with HCC metastasis and recurrence ($p < 0.01$) *(124)*. ZHX2, described earlier as a possible HCC diagnostic marker, was also found by TMA to be expressed significantly higher in primary lesions with metastasis than in those without this phenotype *(74)*. A significant overexpression of clusterin (CLU) was found in metastatic HCC in a paired tissue study ($n = 104$), and Id-1 (inhibitor of differentiation/DNA synthesis) and also Rac and VEGF, key angiogenic factors in cancer progression, were correlated with HCC metastasis by TMAs *(125, 126)*. Meanwhile, aCGH array analysis of early and advanced components of nodule-in-nodule HCC found that genetic inactivation of the APC gene played a significant role in the progression of sporadic HCC, possibly through activation of the Wnt/β-catenin pathway *(127)*. Another study revealed that loss of 17p13.3 and 8q11 was an independent prognostic indicator of poor HCC patient survival *(81)*. LOH has also been observed at 16q and 17q in HCC and occurred more frequently in metastatic lesions *(128)*. aCGH was also used to examine the 7q21-q22 region for its involvement in HCC and found alterations in PFTAIRE protein kinase 1 (PFTK1), ODAG, CDK6, CAS1, PEX1, SLC25A, and PEG10 within this region *(109)*. The authors suggest that upregulation of PFTK1, in particular, may confer a motile phenotype in malignant hepatocytes that correlates with metastasis.

Tumor recurrence complicates resection in a large percentage of cases due to either true metastases or development of de novo tumors.

Vascular invasion, multinodularity, and degree of differentiation are the major predictors of recurrence. Kurokawa et al. identified a 20-gene signature using a PCR-based platform that could predict recurrence with 70% accuracy in an independent cohort of 40 patients *(129)*. A cDNA-based study of 18 HCCs found a 14-gene signature that differed between vascular invasion status and could predict post-resection recurrence *(130)*. cDNA array of HCCs identified claudin-10 expression level to be associated with disease recurrence and was validated by qRT-PCR and associated with survival in multivariate Cox regression analysis *(131)*. In addition, cDNA analyses found gene sets linked to early intrahepatic recurrence including a downregulation of immune response-related genes encoding MHC class II antigens (HLA-DRA, HLA-DRB1, HLA-DG, and HLA-DQA) *(132, 133)*. cDNA arrays have also been used to identify a 46-gene signature associated with extrahepatic recurrence *(134)*. Meanwhile, a 12-gene OLIGO array-based signature has also been shown to predict recurrence within 1-year post-surgery with 93% accuracy *(135)*. A recent follow-up study showed that 3 of these 12 genes (HLA-DRA, DDX17, and LAPTM5) could predict early intrahepatic recurrence with 81% accuracy and were independent risk factors associated with recurrence in a multivariate analysis *(136)*. Another OLIGO study identified a 57-gene signature that could predict recurrent disease at diagnosis with 84% accuracy and was validated in an independent test set *(137)*. The 20-miRNA metastasis signature identified in our laboratory was also significantly associated with recurrence in early-stage HCC *(138)*.

Studies have suggested that while tumor cells affect metastatic capacity, the organ microenvironment can also contribute to this phenotype *(139–141)*. To determine the role of the hepatic microenvironment in HCC metastasis, our laboratory compared the cDNA profiles of noncancerous surrounding hepatic tissues ($n = 115$) from HCC patients with venous metastases which we termed a *m*etastasis-*i*nclined *m*icroenvironment (MIM) sample to those without detectable metastases, which we termed a *m*etastasis-*a*verse *m*icroenvironment (MAM) sample *(138)*. We identified a unique change in the gene expression profiles associated with a metastatic phenotype which was refined to 17 immune-related genes. This signature was inherently different from the HCC tumor signature found in our laboratory and was validated in an independent cohort ($n = 95$). The non-tumor signature could successfully predict venous and extrahepatic metastases by follow-up with >92% overall accuracy and was a superior and independent prognostic indicator when compared with other available clinical parameters for determining patient survival or recurrence. Dramatic changes in cytokine responses, favoring an anti-inflammatory microenvironmental condition, occur in MIM samples, where a predominant Th2-like cytokine profile, favoring a humoral response, was associated with MIM

cases. Colony stimulating factor-1 (CSF1) may be one of the cytokines overexpressed in the liver milieu that is responsible for this shift.

Metastasis and recurrence continue to plague HCC patient outcome. Array profiling methods have identified many alterations that occur in HCC metastasis, some involving well-known metastasis-associated factors such as the angiogenesis-related VEGF and others identifying novel players related to this phenotype. In addition, permissive microenvironments have also been shown to influence HCC metastasis. These metastasis signatures have broadened our knowledge of the biological pathways that are affected during this process and have highlighted particular biomarkers that may be useful to identify HCC patients who are prone to metastasis/recurrence and are tools that can be used to stratify patients for adjuvant therapy. However, the signatures discussed above are largely non-overlapping, suggesting a significant heterogeneity. Although some of these markers have been associated with outcome, future validation and functional/mechanistic studies will be needed to assess their prognostic significance.

3.3. Hepatic Stem Cell Signatures

The heterogeneic nature of HCC and variability of its prognosis suggest that this disease may comprise several distinct biological subtypes. As discussed, microarrays have aided in characterizing separate HCC subtypes with distinct molecular features. Differences in HCC subtypes may arise from activation of different oncogenic pathways during tumorigenesis and/or from different cell origins. Microarray analysis can aid in determining the characteristics of separate HCC subtypes that can provide insight into the cellular origin of the tumor.

Recent studies suggest that HCC may arise from liver stem cells or cells with stem cell-like features which are capable of cellular plasticity, dynamic cell motility, and integral interaction with the microenvironment and are associated with poor outcome. Integrated gene expression data from fetal hepatoblasts and adult hepatocytes with HCC from human and mouse models found that individuals with HCC who shared a gene expression pattern with fetal hepatoblasts had a poor prognosis *(142)*. The gene subset included markers of hepatic oval cells, suggesting that HCC of this subtype may arise from hepatic progenitor cells and analyses of gene networks revealed an activation of AP-1 transcription factors. In our laboratory, we have used cDNA arrays to identify a HCC subtype with features of hepatic stem cells that express AFP and a cell surface hepatic stem cell marker EpCAM *(143, 144)*. EpCAM-positive cells from this subtype have self-renewal and differentiation traits and can initiate highly invasive HCC in NOD/SCID mice (Yamashita et al., unpublished data). The Wnt/β-catenin signaling pathway is augmented in this subtype suggesting that therapeutic approaches geared

toward Wnt/β-catenin signaling inhibitors may impact the survival of HCC patients with this stem cell-like subtype. We have also recently found that miRNAs are associated with this stem cell-like HCC subtype, suggesting that targeting miRNA pathways may alleviate the poor prognosis of HCC patients (Ji et al., unpublished data). However, others have shown that HCC cells that are positive for CD133 or CD90 also have features of cancer stem cells *(145, 146)*. Thus, it appears that hepatic cancer stem cells may also be heterogeneous. It has yet to be determined whether such heterogeneity is due to transformation of different types of stem/progenitor cells or de-differentiation of mature cells.

Recent studies have identified stem cell-like/progenitor cell-like subtypes of HCC that are associated with poor outcome. A clear understanding of these HCC subtypes may identify specific factors that determine more aggressive HCC. Biomarkers associated with these subtypes may help to refine treatment options by allowing more sensitive HCC subtype classification. Furthermore, functional/mechanistic follow-up studies of these stem cell-related biomarkers will aid the generation of novel therapeutic approaches to block pathways associated with poor outcome and thus help to alleviate dismal prognosis.

4. CANDIDATE SERUM MOLECULAR MARKERS

The identification and validation of molecular biomarkers, such as those described above, are relevant toward understanding the pathways that are important for HCC-related disease. Several of these HCC biomarkers have also been associated with diagnosis and prognosis. Importantly, some studies have been validated in independent cohorts and include markers that are expressed in sera, paving the way for clinically useful platforms to assess HCC risk and outcome. Some examples of serum biomarkers identified by HCC array studies are presented below.

4.1. Diagnostic Serum Markers

4.1.1. α-Fetoprotein (AFP)

Since its detection in the serum of HCC patients in 1970s, AFP has been the only serological marker widely used for diagnosing HCC patients. This marker allows for the identification of a small set of HCC patients at an early stage with smaller tumors who have a relatively long-term survival rate following curative treatment *(9, 15, 147)*. Recent array studies have shown that AFP status not only distinguishes HCC from normal but can also be useful in distinguishing HCC subtypes with differing prognostic outcome *(101, 143, 144)*. Although other diagnostic markers have been tested for

HCC diagnosis, without sufficient sensitivity and specificity AFP remains the only universally accepted HCC biomarker in clinical practice. However, non-specific elevation and differences in AFP status among ethnic groups remain to be addressed.

4.1.2. Glypican-3 (GPC3)

Glypican-3 (GPC3) is a member of the glypican family of glycosylphosphatidylinositol-anchored cell-surface heparan-sulfate proteoglycans that interacts with and modulates various growth factors *(148)*. Recent studies indicate that GPC mRNA levels are increased in a large proportion of HCC *(149)*. The level of GPC3 in serum is significantly higher in patients with HCC when compared to healthy patients and is detectable in 40–53% of patients with HCC and in approximately one-third of patients with HCC with normal AFP levels *(150–152)*. Moreover, the expression of GPC3 is independent of the differentiation status and size of HCC *(152)*. In addition, using a cDNA approach, our laboratory has found that an increased expression of GPC3 is associated with most HCC samples including those with normal serum AFP and small tumor size *(54)*. GPC3 was also shown to be upregulated in HCC using cDNA arrays in an independent study showing a link with integrin and Akt/NF-kB pathways *(64)*. This protein is a promising new diagnostic biomarker for HCC.

4.1.3. Midkine (MDK)

Midkine (MDK) encodes a novel heparin-binding growth factor originally identified in embryonal carcinoma cells that is involved in the early stage of retinoic acid-induced differentiation *(153)*. Analogous to AFP, MDK mRNA is highly expressed during embryogenesis but is undetectable in adult tissues except kidney *(154)*. Serum MDK has been reported to be elevated in patients with various types of carcinomas, but not in normal individuals *(155)*. Similarly, an increased expression of MDK has been reported to be associated with HCC *(156, 157)*. Midkine is thought to be involved in carcinogenesis and tumor progression by promoting vascularization, fibroblast growth, and cell migration while suppressing apoptosis *(158, 159)*. In a study performed in our laboratory, MDK was a candidate serum expressed protein that was associated with HCC, including those with normal serum AFP and small tumors *(54)*. These studies suggest that MDK plays an important role in carcinogenesis and the development and metastasis of tumors and that it could serve as a novel tumor marker. Since MDK can be detected in serum, it may be offered as a potentially less invasive diagnostic marker, especially for those who are negative for AFP. Further studies will be needed to validate its use.

4.1.4. Cystatin B (CSTB)

Cystatins are endogenous inhibitors of lysosomal cysteine proteinases *(160)*. Cystatin B (CSTB) is a member of the cystatin superfamily and mutations resulting in a loss of function are responsible for an inherited, progressive, and lethal autosomal disease *(161)*. Furthermore, the activity of CSTB has been reported in several human carcinomas and is overexpressed preferentially in HCC *(44, 162–164)*. In addition, CSTB protein levels were detectable in HCC tumor tissues compared with corresponding non-tumor tissues, and CSTB level was significantly elevated in HCC serum compared with healthy patients or those with chronic liver disease. Therefore, CSTB is specifically overexpressed in HCC tissues and in HCC patients. Whether other CSTB family members are associated with HCC remains to be elucidated.

4.1.5. Complement C3a (C3a)

Complement (C3a) components are important mediators of inflammation and contribute to the regulation of the immune response. Complement activation with subsequent deposition of complement components on tumor tissue has been observed in cancer patients *(165)*. Human C3a is the most abundant complement protein in serum and has been reported to contribute to the early priming stages of hepatocyte regeneration after toxic injury and partial hepatectomy *(166, 167, 168)*. Using proteomic arrays to search for HCC biomarkers, C3a was found to be downregulated in HBV-related HCC *(169)*. Meanwhile, other protein array studies have shown that C3a is specifically upregulated in patients with chronic hepatitis C and those with HCV–HCC, highlighting a difference between HBV and HCC *(6)*. The expression of C3a in HCC sera was further validated by PS20 chip immunoassay and Western blotting. The level of C3a, however, did not correlate with alanine aminotransferase (ALT) values, tumor size, or cirrhosis in chronic hepatitis C and HCV-related HCC groups. Although C3a did not correlate with known clinical parameters, it may be an independent marker for chronic hepatitis C and HCV-related HCC. Taken together, these findings suggested that C3a is associated with the process that leads to the development of HCC.

4.1.6. Insulin-Like Growth Factor (IGF-II)

Insulin-like growth factor (IGF-II) is a mitogenic polypeptide closely related to insulin that serves as an autocrine growth factor in various cancers and is highly expressed during hepatocarcinogenesis *(170, 171)*. It is also associated with the induction of various angiogenesis factors *(172)*. Two comparative studies of AFP and IGF-II serum levels in HCC patients and cirrhotic or normal control subjects found that these two markers were closely

associated in terms of expression and could function as complementary tumor markers *(173, 174)*. IGF-II was increased in HCC patients as compared to cirrhotic and normal controls. In cDNA array studies of 43 different human HCC samples and 3 HCC cell lines in comparison with normal adult liver, two main groups of HCC (designated group A and group B) were identified *(100)*. Based on the expression pattern, group B was further subdivided into two subgroups. A prominent characteristic of subgroup B1 and HCC cell lines was the overexpression of insulin-like growth factor IGF-II. Moreover, IFN-γ treatment substantially reduced IGF-II expression in HCC cells. In a proteomic array study of 210 HCC specimens and corresponding liver tissue, IGF-II was significantly upregulated in HCC and was confirmed by Western blot analysis and TMAs *(175)*. This profiling may be of mechanistic and therapeutic impact because IGF-II overexpression has been linked to reduced apoptosis and increased proliferation and may be accessible to therapeutic intervention. IGF-II may also play an important role in the development of neovascularization and HCC metastasis and may therefore be a useful marker not only for diagnosis but also for prognosis *(176, 177)*.

4.2. Prognostic Serum Markers

4.2.1. Osteopontin (OPN)

Osteopontin (OPN, SPP1) is a secreted multifunctional glycoprotein expressed at high levels in tumors and the surrounding stroma of numerous cancers, including the liver *(178–180)*. Increased serum and plasma OPN levels are associated with advanced-stage lung, breast, colon, and prostate carcinomas *(181–183)*. Importantly, OPN expression can predict high-grade, late-stage, and early-recurrence HCC and is highly correlated with tumor recurrence and decreased patient survival following orthotropic liver transplantation *(184)*. OPN was also shown to be upregulated in HCC using cDNA arrays in an independent study showing a link with integrin and Akt/NF-kB pathways *(64)*. In our laboratory, we have shown that OPN is a significant factor in HCC metastasis *(122)*. Similar findings have been shown in metastatic tumor cell lines and breast cancer patients *(185–187)*. Furthermore, a neutralizing antibody to OPN can decrease pulmonary metastases in nude mice and inhibit tumor cell invasion, highlighting an essential role of OPN in HCC metastasis *(122)*. We have also found that elevated expression of OPN is concordant with matrix metalloproteinase-9 (MMP-9) in primary metastatic HCC *(188)*. We found that MMP-9 cleaved OPN into specific fragments, one of which (OPN-5kD, residues 167–210) could induce low-metastatic HCC cellular invasion via CD44 receptors which was effectively blocked by the addition of small peptides within the region of OPN-5kD. In addition, increased expression of an OPN splice variant (OPN-c) was

associated with clinical metastatic HCC. Thus, a distinct region of OPN was shown to be most essential for HCC cellular invasion and appears to correlate with their metastatic potential. Our data also suggest an alternative splicing event (OPN-c) promotes extracellular cleavage of OPN by MMP-9 to release OPN-5kD. These findings may help to improve advanced-stage HCC prognosis and suggest the utility of small peptides for novel therapies.

4.2.2. Colony Stimulating Factor-1 (CSF1)

Macrophage colony stimulating factor (CSF1), originally identified as a hematopoietic growth factor, is a dimeric polypeptide growth factor that acts through the cell surface receptor (CSF1R) that stimulates proliferation, differentiation, and survival of monocytes and macrophages *(189)*. CSF1 was originally identified as a regulator of the proliferation, differentiation, and survival of macrophages and their bone marrow progenitors *(190)*. However, in addition to its normal role in mononuclear phagocyte biology, elevated expression of CSF-1 and *cfms* has been found in breast, uterine, and ovarian tumor cells, and the extent of expression in these tumors correlates with high grade and poor prognosis *(191–193)*. The biological role and possible clinical significance of these macrophages are still unknown and remained controversial. Studies have shown that macrophages can serve as both positive and negative mediators of tumor growth. Macrophages are known to mediate direct antitumor cytotoxicity and the presentation of tumor-associated antigens *(194)*. On the other hand, macrophages have also been found to promote tumor angiogenesis and to secrete a wide range of growth factors which may promote tumor growth *(195)*. However, as most of these data are derived from studies of cultured tumor cells or from clinical observations, the functions for macrophages in the tumor microenvironment have still not been determined.

In HCC, we have shown that a unique inflammation/immune response-related signature is associated with noncancerous hepatic tissues from metastatic HCC patients and is principally different from that of the tumor. A global Th1/Th2-like cytokine shift in the venous metastases-associated liver microenvironment coincides with elevated expression of CSF1. A refined 17-gene signature containing CSF1 was validated as a superior predictor of HCC venous metastases in an independent cohort, when compared to other clinical prognostic parameters. Our results show that the T cell population may be involved in the promotion of Th2 cytokines and repression of Th1 cytokines in peripheral blood mononuclear cells (PBMC) induced by CSF1. It is possible that these T cell populations are differentially primed in pro-metastatic conditions, in part by the activity of CSF1, and thus produce cytokine profiles that favor cancer advancement. We suggest that a predominant humoral cytokine profile occurs in the metastatic liver milieu and a

shift toward anti-inflammatory/immune-suppressive responses may promote HCC metastases.

4.2.3. Vascular Endothelial Growth Factor (VEGF)

Angiogenesis is a neovascularization process essential for tumor growth, invasion, and metastasis *(196, 197)*. Angiogenesis is regulated by various angiogenic factors of which vascular endothelial growth factor (VEGF) seems to play a central role *(198)*. The elucidation of the mechanisms of angiogenesis is of importance because anti-angiogenic agents are now available and may be of potential benefit in patients with HCC *(199)*. VEGF overexpression and increased serum level has been associated with a greater risk of metastasis, recurrence, and poor survival in HCC *(200–202)*. VEGF was among the top angiogenic factors expressed in HCV–HCC tissues in an OLIGO array study compared to normal livers *(50)*. Moreover, VEGF was also differentially expressed when HCV–HCC samples were compared to HCV cirrhotic tissues. In a TMA study, it has also been shown that Id-1 (inhibitor of differentiation/DNA synthesis), which belongs to the Id family of helix–loop–helix proteins, might enhance HCC angiogenesis and metastasis through interaction with VEGF *(126)*. Therefore, soluble angiogenic factors, such as Id-1 and VEGF, might be useful for monitoring high-risk HCV patients and might be novel targets to inhibit HCC metastasis through suppression of angiogenesis.

4.2.4. Angiopoietins (Ang-1 and Ang-2)

Angiopoietins (Ang) are endothelial cell growth factors which act as ligands for the tyrosine kinase receptor, Tie2. The Ang-1/Tie-2 pathway is thought to mediate the vital functions of vascular stabilization and vascular remodeling, via integration of periendothelial cells into the vascular wall, particularly in the presence of VEGF. In contrast to Ang-1, Ang-2 induces vascular regression in the absence of VEGF but increases vascular sprouting in its presence *(203)*. Overexpression of Ang-2 has been associated with poor prognosis and reduced disease-free survival in several human cancers, including HCC *(204)*. It has been shown that the ectopic expression of Ang-2 in HCC cells promotes rapid development of tumor and aggravates its prognosis, suggesting that the Ang-2/Tie-2 pathway might be involved in angiogenesis of HCC. Thus, increased expression of Ang-2/Tie-2 appears to play a role in promoting tumor angiogenesis in human HCC *(205, 206)*. In a human angiogenesis OLIGO array, Ang-1 and Ang-2 were overexpressed in HCV–HCC *(50)*. In addition, serum levels of Ang-2 were found to be elevated in patients with cirrhosis and more so in HCC *(205)*. Thus, monitoring the serum level of angiogenic factors may be helpful in clinical recommendations for HCC.

4.2.5. Fibroblast Growth Factor (FGF)

Fibroblast growth factor (FGF) is a soluble heparin-binding polypeptide with a potent mitogenic effect on endothelial cells. The upregulation of FGF has been associated with tumor metastasis and recurrence in HCC *(124)*. In a TMA study, FGF was shown to be elevated in HCV–HCC samples *(50)*. In separate studies, serum FGF-2 was significantly elevated in patients with HCC compared with healthy volunteers and circulating basic FGF plasma levels were an indicator of CLD progression *(207, 208)*. The prognostic significance of serum FGF following resection for HCC was evaluated by Poon and colleagues who found that high levels of FGF independently predicted decreased disease-free survival on multivariate analysis in a series of 88 patients *(209)*. This finding indicates that upregulation of FGF may play an important role in HCC metastasis and recurrence. Further study of FGF may provide a new insight to evaluate HCC metastasis and prognosis.

4.2.6. Hepatocyte Growth Factor (HGF)

Hepatocyte growth factor (HGF) is a multifunctional cytokine that affects mitogenesis, cell motility, matrix invasion, and epithelial carcinogenesis *(210)*. In a human angiogenesis OLIGO array, HGF was found to be overexpressed in HCV–HCC *(50)*. Several reports have shown increased serum HGF levels in patients with chronic hepatitis infection and HCC *(211–214)*. High HGF concentrations were associated with a significantly increased risk of HCC development and some studies have shown an association with tumor metastasis and poor prognosis after hepatic resection *(215–217)*. Hepatocyte growth factor may, therefore, be a target of future HCC postoperative treatment. Additional studies will be needed to determine whether inflammatory changes rather than hepatic carcinogenesis are responsible for increased serum HGF levels in patients with chronic hepatitis and HCC.

4.2.7. Interleukin-6 (IL-6)

Interleukin-6 (IL-6) is cytokine associated with the inflammatory process. Although considered to be hepatoprotective *(218)*, it has also been shown that persistent high levels of IL-6 causes liver damage *(219)*. In an OLIGO-based microarray study of HCV core-infected hepatocytes, IFN-stimulated genes were increased, including IL-6 *(220)*. The authors suggested that IL-6 could play a role in modulating cell growth through alterations in Stat3 signaling and regulation of c-myc and cyclin D. Other studies have shown that IL-6 levels increase upon both HCV infection and expression of HBx *(221, 222)*. The circulating serum level of IL-6 has been associated with many cancer types and was shown to correlate with invasion and metastasis *(223)*. In HCC, higher serum IL-6 was observed in comparison to patients with cirrhosis or normal controls and was significantly more discriminate than AFP *(224)*. In a study of 80 HCC patients, however, IL-6 serum levels

did not correlate with outcome *(225)*. Kupffer cells, the liver macrophages, express IL-6; however, various human tumor cells can produce IL-6 and thus affect disease severity *(226)*. Since IL-6 is involved in HCC progression, this cytokine may be useful as both a diagnostic and a prognostic marker. Further studies will be needed to validate these findings.

Thus, several serum-based biomarkers have been identified from array-based studies. Interestingly, biomarkers associated with inflammation and angiogenesis have been predominantly found to be associated with HCC prognosis, reinforcing the importance of changes in the immune system and phenotypes of metastasis on patient outcome. AFP, however, still remains the most sensitive and specific biomarker for HCC diagnosis and prognosis. Improvements in measurement and perhaps combinatorial studies will provide more sensitive/specific biomarkers in the future. These examples of diagnostic and prognostic serum markers, however, are notable advances in the application of information gained from array-based studies toward clinical practice.

5. SUMMARY

The advent of microarray technology has provided a high-throughput methodology to assess the genome-wide changes that occur during hepatocarcinogenesis. Using multiple sample types, array platforms, and data analysis methods, the mechanisms related to HCC carcinogenesis can be elucidated and related to disease pathogenesis and clinical measures. The definition of molecular markers from these studies has the potential to revolutionize the diagnosis and prognosis of patients with HCC.

Microarrays have steadily become more comprehensive and stable, not only increasing the number of elements that can be arrayed but also expanding with regard to the types of material that can be analyzed. Despite advances in stability and composition of microarrays, several fundamental issues still remain to be resolved. These include multiple sources of variation (among samples, within arrays, mixed cell types, user-related error, etc.) which may lead to overinterpretation or spurious functional gene associations. In addition, the need for physical destruction of cells/tissues limits consequential assays conducted on the same material. Advanced techniques such as laser capture microdissection and automation have somewhat improved these challenges. The overall quality and amount of starting material is a major challenge and is limited by the amount and complexity of the sample as well as user-related handling. In addition, many oncogenic processes are not accounted for by array analysis since they are regulated post-transcriptionally. Therefore, elements such as protein localization and modification need to be included in HCC profiling. Difficulties in data comparison must also be addressed which ensues from the use of multiple array

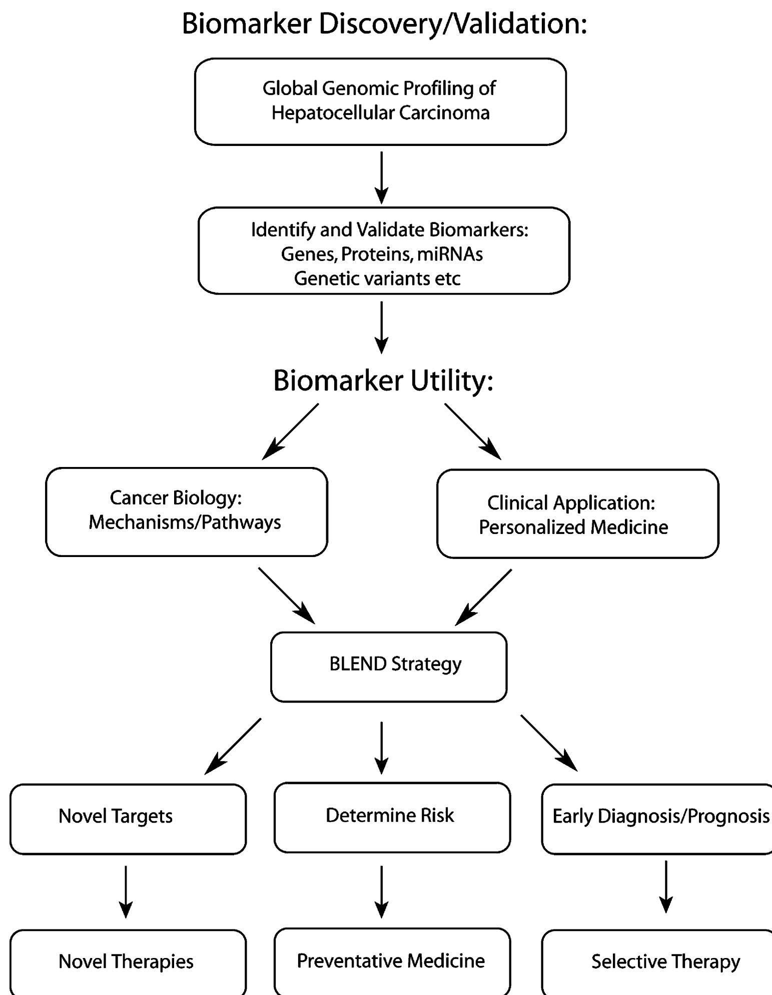

Fig. 1. Global expression-based biomarker identification, validation, and clinical utility. Wide-screen genomic profiling of hepatocellular carcinoma (HCC) has identified multiple biomarkers on the gene, protein, and genomic scale. These biomarkers are useful for understanding HCC biology and clinical application. The mechanistic and clinical information gleaned from genomic profiling studies can be combined using a Biological Expression Network Discovery(BLEND) strategy to identify promising novel therapeutic markers for diagnosis, treatment, and prognosis of HCC. Such methods will allow progression toward personalized medicine encompassing new and selective therapeutics and preventative therapy.

platforms and data algorithms among published studies as well as frequent updates of genomic databases. Such problems may be alleviated by setting adherence guidelines for microarray statistical analysis and reporting such

as those established by the International Microarrays Gene Expression Data group, the REMARK guidelines, or incorporation of proper study design that is suitable for array-based biostatistical analyses *(227–229)*. Resolution range is a large limitation in array analysis, whereby important changes may not be assessed or studied due to the cutoff criteria in the analysis. In addition, each microarray can only provide information concerning the targets that are included on that array. Future studies may require integrative analysis of multiple platforms in order to define the exact cancer-related molecular changes on multiple biological levels and to distinguish the key players from their downstream effects. Advancement in statistical methods to integrate multiple platforms will be required to make such assessments. Recently, systems have been developed (e.g., Illumina Genome Analyzer) that offer whole-genome analysis using a massive parallel sequencing that is useful for discoveries in genomics, epigenomics, gene expression, and protein–nucleic acid studies. Such systems offer an extremely high-throughput method to complete large-scale global studies in an accurate manner and may allow for ease in cross-platform-type analyses since an enormous multilevel data set can be achieved with a relatively small amount of the same starting material. The utilization of a Biological Expression Network Discovery (BLEND) strategy integrating global molecular profiling data along with mechanistic/functional studies may improve the diagnosis, treatment, and prognosis of HCC patients (Fig. 1).

Although multiple publications have identified and validated diagnostic and/or prognostic HCC markers (Table 2), critical challenges in translating the findings to clinical practice remain. To reach clinical applicability, the measurement of biomarkers must be reproducible, reliable, and easily accessible by non-invasive methods. In addition, the biomarker sets will need to be refined to a smaller number of informative biomarkers to be useful for clinical interrogation. Large prospective studies will need to be performed to assess appropriate sample size for accurate diagnostics and appropriate validation cohorts will be needed to incorporate gender, race, and underlying etiological differences among HCC patients. Nonetheless, the biomarkers that have been identified through gene profiling, particularly those expressed in serum, are an unprecedented advance toward useful clinical application.

Overall, molecular profiling studies have become powerful methods to incorporate global genomic readouts with biological effects and are conduits for the discovery of biomarkers with potential clinical application. The HCC-related genomic expression studies presented in this chapter along with future studies and advances in microarray technology, experimental design, and statistical analyses will undoubtedly lead to crucial and important progress in our understanding of the molecular mechanisms and biology of HCC. Moreover, these studies have revealed molecular markers that provide the framework toward predictive and personalized care for HCC

Table 2
HCC Clinical Markers Defined by HCC Microarray

Diagnosis/prognosis		*Platform*	*Prediction signatures*	*Top five significant genes/miRNAs/proteins*	*Ref*
Diagnosis	**Early HCC**	**cDNA**	25 genes	Not listed	*(44)*
			40 genes	Not listed	*(104)*
		Proteome	6 proteins	DEAD box polypeptide 3, eEF2, AIF, hnRNP A2, prostatic-binding protein, TIM	*(242)*
	HCC subtypes	**cDNA**	83 genes	*ACP5, RPL39L, TACSTD1, H1F0, H19* in HBV-HCC; *C1S, IFI27, TAT, AZGP1* in HCV-HCC	*(45)*
			59 genes	Not listed	*(62)*
			HCC subtype genes	Not listed	*(100)*
			2 genes	*EpCAM, AFP*	*(144)*
	Staging	**cDNA**	240 genes	Not listed	*(105)*
			31 genes	*IQGAP1, PPT1, CTSC, LIPA, SNX2*	*(103)*
	HCC markers	**cDNA**	50 genes	*PGCP, PLA2G13, and PLA2G7*	*(58)*

Table 2
(Continued)

Diagnosis/prognosis		*Platform*	*Prediction signatures*	*Top five significant genes/miRNAs/proteins*	*Ref*
			68 genes	Not listed	*(61)*
			44 genes	Not listed	*(59)*
			63 genes	Not listed	*(102)*
			217 genes	Not listed	*(115)*
			5 genes	*GPC3, PEG10, MDK, SERPINI1, QP-C*	*(54)*
			248 genes	*EIF3S3, C9, UBD, FLJ42752 fis, SLC12A8*	*(239)*
		microRNA	8 miRNAs	miR-18, miR-199a∗, miR-199a, miR-224, miR-195	*(89)*
			40 miRNAs	Not listed	*(241)*
		Proteome	250 features	Not listed	*(97)*
			4 peptides	7486, 12843, 44293, 53598 Da	*(99)*
			11 peaks	Cystatin C	*(98)*
Prognosis	**Metastasis**	**cDNA**	90 clones	Not listed	*(114)*
			30 genes	Not listed	*(122)*
			35 genes	*HFL3, PMS2L11, SGK, GPRK6, ZNF216*	*(238)*
			17 genes	Not listed	*(138)*

(*Continued*)

Table 2
(Continued)

Diagnosis/prognosis		*Platform*	*Prediction signatures*	*Top five significant genes/miRNAs/proteins*	*Ref*
		microRNA	20 miRNAs	miR-30c-1, miR-1-2, miR-34a, miR-148a, miR-124a-2	*(123)*
	Recurrence	**cDNA**	3 genes	*HLA-DRA*, *DDX17*, *LAPTM5*	*(135, 136)*
			20 genes	*ALCAM*, *FLJ37965*, *NRG2*, *CDH1*, *RGS5*	*(129)*
			4 genes	*HLA-DRA*, *HLA-DRB1*, *HLA-DG*, *HLA-DQA*	*(235)*
			36 genes	*TRIM25*, *EIF2S3*, *CLECSF14*, *DXYS155E*	*(60)*
			14 genes	Not listed	*(130)*
			46 genes	*MPV17*, *GZMB*, *ITGA6*	*(134)*
			57 genes	*USH1C*, *CNGA1*, *INSIG1*, *RACGAP*, *GSTM3*	*(137)*
	HCC risk	**cDNA**	30 genes	*CP*, *CACNB3*, *MT1E*, *DYRK3*, *UAP1*	*(90)*
	Markers	**Genome**	2 regions	Loss on 17p13.3; Gain on 8q11	*(81)*
		cDNA	406 genes	Not listed	*(119)*
			Angiogenesis soluble factors	Not listed	*(50)*

patients. We are now at the brink of clinically implementing biomarkers identified from global gene expression profiling to improve HCC diagnosis, treatment, and outcome.

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