Molecular Profiling of Human Hepatocellular Carcinoma

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5.1 Hepatocellular Carcinoma: Clinical Concerns

The wide heterogeneity of HCC and the complexity of its diagnostic and prognostic assessment (dependent upon tumor grade/residual liver function contributed by various etiological factors) 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 [1–6]. Although individuals at high risk for HCC development are routinely screened by ultrasonography and serum alpha-fetoprotein (AFP), most patients are diagnosed at advanced disease stages. AFP evaluation however, can be nonspecific, vary significantly between ethnic groups and is only observed in a HCC subgroup with small tumors [7]. 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 [8–10]. 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 renders HCC treatment especially difficult [11]. 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 postsurgical survival is complicated by a predom-

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inant occurrence of tumor recurrence/metastasis [12–14]. Methods to improve survival include percutaneous ethanol injection, radiofrequency ablation, and transarterial chemoembolization (TACE) [15, 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.

5.2 Molecular Profiling: Technologies and Platforms

The gene expression profile of a particular cell type or tissue has been analyzed in earlier years 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), metabolites (metabolomics), and genetic aberrations (array CGH/methylation) [17–19]. In addition, sequencing on the DNA and RNA level has also increased our capacity to identify the mutation landscape of HCC [20–22]. Although previous methodologies to study HCC have advanced the field, molecular profiling of clinical samples from HCC patients and HCC-related cell lines have enriched the breadth of HCC knowledge and have allowed researchers to begin to tackle some of the key disease-related concepts that still remain.

5.2.1 Molecular 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 multiple layers of signaling information including the genome, epigenome, transcriptome, proteome, and metabolome. A brief overview of widely used array platforms is provided below.

5.2.1.1 Genomic Profiling (aCGH, Methylation, Sequencing)

Array Comparative Genomic Hybridization

An important method of identifying driver genes involved in HCC is to detect genomic regions that undergo frequent alterations or are modified. Several types of alterations are present in the liver including changes in gene copy number, mutations, and chromosomal rearrangements. Array Comparative Genomic Hybridization (aCGH) using the BAC-based (Bacterial Artificial Chromosome) and oligonucleotide-based CGH enables high-resolution multi-loci mapping of small genomic regions with copy number changes, such as amplification or deletion [23, 24]. BAC aCGH is limited by costly, time-consuming, low-yield clone production and noisy data due to nonspecific hybridization of repetitive sequences. Oligonucleotide aCGH allows for flexibility in probe design, greater genomic coverage, and higher resolution ($\sim$50 kB). 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 requires a high concentration of high-quality BAC DNA for good array performance [25, 26]. Recently, genome-wide approaches, such as the single nucleotide polymorphism (SNP) 6.0 arrays, have allowed for global analyses of copy number alterations in HCC. Using these methods, numerous amplified and deleted genes have been observed in HCC.

Methylation

A few CGH array studies have been followed by bisulfate DNA sequencing or methylation-specific PCR to identify HCC-related epigenetic changes [27–29]. Since HCC develops against a background of chronic liver damage, the extent of genetic and epigenetic alterations is essential for our understanding of this cancer. In particular, methylation at CpG sites in gene promoters can affect the transcription of important genes in cancer. In fact, several hypermethylation events have been observed in tumor suppressor genes, suggesting a role for carcinogenesis promotion via this disruption of normal transcriptional events and induction of chromosomal instability. Indeed, certain methylation events have been associated with HCC patient survival and

recurrence and targeting of the epigenetic machinery has been the basis of some trials for HCC therapy [30, 31]. Methylation events can occur in several sites, including gene promoters, gene bodies, repetitive sequences, and intergenic regions, however the functional importance of specific alterations currently remains unclear. The induction of methylation events is also largely unknown, although some studies have shown that hepatitis viral infections, as well as nonalcoholic fatty liver disease can induce changes in methylation [32, 33]. Comprehensive methylation profiles of HCC are now readily studied by array-based platforms such as the Human Methylation 450 Bead Array and next-generation sequencing technology [34]. Our understanding of the HCC epigenetic code may allow for the development of novel diagnostic and therapeutic approaches for HCC.

Sequencing

High-resolution assessment of the liver cancer genome is now possible through advances in next-generation sequencing technologies [35]. An in-depth exploration of the liver genome has recently been employed through whole exome sequencing. This method is based on the capture or enrichment of DNA fragments containing the exonic region followed by massively parallel sequencing to determine somatic mutations [36–38]. Using this technology, several somatic alterations in the protein-coding region have been identified in HCC. To further identify somatic drivers in HCC, efforts have also been made to sequence the entire liver genome. This is referred to as whole genome sequencing whereby structural rearrangements, substitutions in noncoding regions, and viral integration sites can be explored. These methods however, look at rather short lengths of DNA sequences and thus, the identification of large genomic alterations is still rather limited. Although several key molecules have been indentified or validated by these methods, there seem to be a large number of passenger mutations present, which makes the identification of key driving genes in HCC a more complex problem.

RNA sequencing meanwhile, has added to our capacity for transcriptome profiling by allowing us to explore rearrangements in transcripts, noncoding RNAs, and splicing events. This highly sensitive method provides a more accurate tool for measuring expression across the transcriptome. Transcript abundance is quantifiable using this method, along with the identification of both known and novel features in the coding and noncoding transcriptome. Overall quality of starting samples, sequencing libraries, sequencing coverage, as well as time and cost parameters can have a significant impact on the sensitivity of detection and data quality in these types of experiments. These comprehensive genomic analyses however, are enabling researchers to examine the liver cancer genome at a much higher resolution with potentially impactful findings that could advance clinical management of this disease.

5.2.1.2 Transcriptomic Profiling (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 [39]. Multiplexed target profiling of hundreds of transcripts is also readily available through newer applications such as Nanostring [40]. In addition, the regulation of mRNAs can be analyzed using noncoding arrays (e.g., microRNA, pre-microRNA, snoRNA), which globally interrogate the expression of small endogenous (21–35 nt) RNA species. Platforms that detect mature and precursor forms of >2000 miRNAs are now commercially available [41–43].

5.2.1.3 Proteomic Profiling (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 posttranslational modifications, and correlate proteins with disease advancement or with certain treatments/environments [44]. Tissue microarrays (TMA) allow tissue-based profiling using small cylinders of formalin-fixed tissues arrayed in a single paraffin block [45]. 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 may help to resolve this problem [46]. Comprehensive proteomic characterization has been performed for certain cancer types, such as colon and rectal, however there is currently a lack of such studies for HCC [47].

5.2.1.4 Metabolomic Profiling

Cancer metabolite profiling (metabolomics) is a promising new approach to understand the biological mechanisms underlying cancer development and progression. Metabolomics provides a global view of metabolites, the

biochemical end products of cellular processes, enabling the characterization of cancer through metabolic changes, whose regulation are tightly linked with a certain pathological state [48]. In fact, metabolites are the best molecular indicators of cell status, since metabolic fluxes can change in a matter of seconds versus the comparatively slower turnover of mRNA and proteins [49, 50]. Thus, metabolic alterations are an extremely sensitive measure of cellular phenotype. Although genomics-based studies have been performed to extensively profile human tumors [51–56], relatively little is known about the global metabolite alterations that characterize cancer and how all of these events are intertwined as a network leading to aggressive disease and poor outcome. A systematic assessment of the pathways in which these genes and biochemical molecules contribute may lead to a more precise set of alterations that may serve as key biomarkers or drug targets for clinical interrogation in cancer patients suffering dismal prognosis.

5.2.2 Computational Analysis

Methodologies for analysis of large-scale omics data can be either unsupervised or supervised [57, 58]. Unsupervised methods attempt to characterize the components of a dataset without a priori input or knowledge of a training set. Internal structure or relationships in datasets are found by feature determination which groups genes/molecules with interesting properties (principal component analysis), cluster determination which groups genes or samples with similar patterns of gene/molecule expression or abundance (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 molecular features that fit a predetermined pattern [59]. This technique finds genes/molecules with expression or abundance levels that are significantly different between groups of samples (e.g., cancer classification) and can be used to find genes/molecules that accurately predict a characteristic of that sample (e.g., survival or metastasis). The significance found by supervised methods has been evaluated using parametric, nonparametric, and analysis of variance procedures which involve permutations, random partitioning of the studied dataset, 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 or abundance, including absolute or ratio of expression or abundance 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. Corrective statistics are also used when identifying genes/molecules of interest, to account for multiple testing in large datasets, including adjusted *p*-value, false discovery cutoffs, and Bonferroni corrections [60]. Due to the high complexity and sheer magnitude of current datasets, such as those ensuing from sequencing studies, new techniques and methods are constantly being explored, updated, and created to adequately analyze data. Many of these methods rely on algorithms and codes, most based on the R programming language, and in-house or stand-alone software associated with new technologies. A gold standard has been proposed for analysis of array studies which involves the use of a training dataset to initially identify a signature, a test dataset to assess its predictive/classification capacity, and an independent set for validation studies [61–63]. Importantly, biomarkers and signatures of interest need to not only be tested in retrospective cohorts, but also in prospective studies and in context of therapeutic strategies for HCC.

5.3 Tumor Signatures

Array studies have provided vast amounts of information concerning the genes, proteins, metabolites, and genomic changes that occur in HCC-related disease. These investigations have revealed changes that occur across the spectrum of cirrhosis, HCC tumors, the HCC microenvironment, HCC subtypes, epigenetic alterations, and progressive phenotypes (metastasis/recurrence). A general overview of these studies along with a synopsis of emerging perspectives gleaned from these analyses is provided in this section.

5.3.1 Tumor-Based Diagnostic HCC Signatures

5.3.1.1 Tumor Biomarkers (Tumor Vs. Nontumor)

Array studies have enhanced our understanding of how the HCC process alters the regulatory network of genes, proteins, metabolites, and epigenetic effects, in a way that differs from the respective normal tissue or disease-free samples. For example, cDNA analysis of HCC versus normal samples have found 38 differentially expressed genes while HBV-related cell lines revealed signatures (356 genes) composed of upregulated ribosomal-related genes [64, 65]. TIPUH1, a regulator of transcription and RNA processing of growth control genes has also been shown to be upregulated in HCC by cDNA array [66]. It has also been 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 [55]. A cDNA array of non-HBV/HCV-infected HCC versus normal tissues revealed 61 differentially expressed genes [67]. 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 [68–70]. Integrin and Akt/NFKB signaling were also upregulated in HCC along with a serum biomarker (CSTB) using cDNA arrays [71, 72]. Similar studies have shown that activators of neutrophils, anti-apoptotic genes, interferon response genes and proteins related to cell differentiation or development are differentially expressed in HCV-HCC [73]. 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 [74]. Several of these pathways, along with growth factor alterations were found in cDNA arrays comparing HBV or HCV-positive tumor versus nontumor tissue [75]. A clear distinction was found between HBV and HCV samples, where HBV affected genes 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 versus nontumor 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 [76].

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 nontumor tissue [77]. Other HCC-related protein classifiers include those involved in heat shock response, glycolysis, fatty acid transport and trafficking, amino acid metabolism, cell cycle regulation and cell stress, and metabolism related enzymes [78–80]. 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 [81]. Quantitative proteomics revealed that the SET complex is associated with HCC [82], while complement C3a was suggested as a HCC biomarker in HCV-HCC [83]. Serum monocyte chemoattractant protein-1 and prolactin have also been identified as potential tumor markers in HCC [84]. A TMA study of HCC versus nontumor found HCC-specific expression of the transcription repressor Zinc fingers and homeoboxes 2 (ZHX2) protein expression which correlated with differentiation stage [85].

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 [86, 87]. In addition, a study of 120 HCC samples found LOH at 6q and 9p in small, well-differentiated tumors [88]. A comparison of tumor versus nontumor 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 [87]. In a study of 20 HCC cases, oncogenes were amplified in 1q, 8p, and 11q regions while loss occurred at 13q and 4q [89]. A study of HCV-HCC revealed that increases of DNA copy number were frequent at 10p while decreases were frequent at 10q [86]. 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 HBV-HCC, gains on 1q, 6p, 8q, 9p were observed while losses in 1p, 16q, and 19p occurred in most patients [90]. 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 [91]. 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) [92, 93].

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 [94, 95]. miRNA array studies have also demonstrated that miR-21 can contribute to HCC growth and spread by modulating PTEN [96]. 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 [97–99]. In another study comparing HCC samples and adjacent nontumor, 8 miRNAs were shown to be significantly altered, 5 of which were downregulated in HCC and could predict HCC with 97 % accuracy [100]. More recently, microRNAs present in the circulation have also been identified as potential biomarkers for HCC [101, 102].

DNA methylation-based prognosis and epidrivers for HCC have also been studied. Villaneuva et al. identified a signature of 36 DNA methylation markers that predicts HCC patient survival and harbor mRNA signatures of tumors with progenitor cell features [103]. Deng et al. applied methylated DNA immunoprecipitation to identify 15 genes preferentially methylated in HCV-HCC [104]. Using a 27 K

Infinium array, thousands of differentially methylated genes in HCC were found, several of which could be assayed in plasma [34]. Tumor from nontumor specimens could be readily indentified in a methylation study of HCC using a 450 K array. Methylation events in p53, CTNNB1, GSTP1, MGMT, RASSF1A and in promoter CpG islands of CDKN2A have also been identified in HCC [105–108].

Array-based comparisons have also been made between early neoplastic stages (fibrosis/cirrhosis) and HCC. A study of 59 preneoplastic chronic liver diseases (CLDs) including hepatitis, autoimmune hepatitis, primary biliary cirrhosis found genes associated with high or low risk of HCC development [109]. This 273-gene signature was validated in three independent cohorts and included 12 secretory genes in the top geneset. 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 [110]. In an OLIGO array-based study of fibrosis, carbohydrate metabolism genes were elevated in HCC patients when compared to cases with F3–4 fibrosis [111]. 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 [112]. An RT-based study of cirrhosis versus HCV-HCC showed that eight genes were significantly altered (GPC3, TERT, Survivin, XLKD1, and CDH1) [113]. MiRNA platforms have also demonstrated that 35 miRNAs including let7 and miR-181 family members differ between HCC and cirrhosis [114]. Circulating microRNAs have also been shown as important modulators in early stage HCC [115]. aCGH of 63 HCCs found etiology-dependent copy number gains, including 8q24 and MYC overexpression in viral and alcohol-related HCCs [116]. 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 [117, 118]. In other studies, and CD5L and Annexin A2 were found as discriminative candidates in HCC [119, 120].

5.3.1.2 Tumor Biomarkers (Epigenetic Signatures)

HCC development is thought to be a multistep process, not only involving accumulation of genetic changes, but also epigenetic changes, such as methylation, which can reversibly alter regulatory genes. Several 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 [121]. In another cDNA array and bisulfite PCR study, insulin-like growth factor binding protein was found to be hypermethylated and downregulated in HCC [122]. 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 [123]. In addition, Pang et al. found a loss of an unmethylated 6q allele in HCC encoding a putative tumor suppressor gene [124]. 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 [125]. A promoter methylation study of 30 HCC tumors showed that they exhibit specific DNA methylation signatures associated with major risk factors and tumor progression stage, with potential clinical applications in HCC diagnosis [126].

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. Across platforms however, marker sets are quite different from one another, despite a similarity in comparison groups which could be due to platform makeup, sample heterogeneity, etiological differences, or ethnicity among samples. In addition, many of these studies lack validation and are only drawn from relatively small datasets 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.

5.3.2 Tumor-Based Prognostic Signatures

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 [127]. In HCC however, such stepwise and specific progression-related genetic changes have not been illustrated.

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 [128]. 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 [129, 130]. 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 [131]. 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 [132]. Another cDNA study of HCC found 217 genes associated with differentiation status and metastasis, including ANXA2 [133]. Another cDNA-based study found that HCC with high expression of ubiquitin-cojugating 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 [134, 135]. 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 [136, 137]. cDNA arrays have also been used to show that intrahepatic metastatic lesions are indistinguishable from their primary HCC while primary metastasis-free HCC was distinct from primary HCC with metastasis [53]. 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. It has also been investigated whether certain miRNAs are associated with HCC metastasis [138]. 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 ($p < 0.01$) [139]. 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 [85]. 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) as well as Rac and VEGF, key angiogenic factors in cancer progression, were correlated with HCC metastasis by TMAs [140, 141]. 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/beta-catenin pathway [142]. Another study revealed that loss of 17p13.3 and 8q11 were independent prognostic indicators of poor HCC patient survival [143]. LOH has also been observed at 16q and 17q in HCC and occurred more frequently in metastatic lesions [144]. The authors suggest that upregulation of PFTK1, in particular, may confer a motile phenotype in malignant hepatocytes that correlates with metastasis. Proteomics has also been applied to understand HCC progression. Tan et al. recently used comparative proteomics to identify proteins to differentiate patients who relapse from those who do not [145]. Proteomics has also been used to identify Talin-1 upregulation to be associated with HCC prognosis [146].

Tumor recurrence complicates resection in a large percentage of cases due either to 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 [147]. A cDNA-based study of 18 HCCs found a 14-gene signature that differed between vascular invasion status and could predict postresection recurrence [148]. 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 [149]. Meanwhile, a 12-gene OLIGO array-based signature has also been shown to predict recurrence within 1 year postsurgery with 93 % accuracy [150]. 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 was an independent risk factor associated with recurrence in a multivariate analysis [151]. 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 [152]. 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) [153, 154]. cDNA arrays have also been used to identify a 46-gene signature associated with extrahepatic recurrence [155]. The 20-miRNA metastasis signature identified was also significantly associated with recurrence in early stage HCC [138].

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 nonoverlapping, 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.

5.4 Microenvironment Signatures

Studies have suggested that while tumor cells affect metastatic capacity, the organ microenvironment can also contribute to this phenotype [156–158]. To determine the role of the hepatic microenvironment in HCC metastasis, the cDNA profiles of noncancerous surrounding hepatic tissues (n = 115) from HCC patients with venous metastases, termed a metastasis-inclined microenvironment (MIM) sample to those without detectable metastases, termed a metastasis-averse microenvironment (MAM) sample were compared [54]. A unique change in the gene expression profiles associated with a metastatic phenotype was identified which was refined to 17 immune-related genes. This signature was inherently different from a signature found in HCC tumor tissues and was validated in an independent cohort (n = 95). The nontumor 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 to 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. Gene expression profiling of nontumor specimens from HCC patients was also used to identify a molecular signature from formalin-fixed paraffin-embedded tissues. This poor prognosis signature was related to impaired liver function and inflammation, particularly interleukin-6. In addition, Hoshida et al. demonstrated that profiles of the surrounding nontumoral liver tissue were highly correlated with survival among Japanese, US, and European patients with HCC [159, 160]. These findings help to solidify the role of the field effect, whereby environmental exposures may play a role in tumor development and progression.

It has also been demonstrated that the expression levels of certain small RNAs, termed microRNAs, are altered in HCC metastasis. In a follow-up study, this 20-microRNA signature was validated and the role of a particular microRNA, let-7g in HCC progression, was determined [161]. It was confirmed that the level of let-7g was significantly lower in metastatic compared to nonmetastatic HCC and was predictive of poor survival. Functional studies indicated that let-7g could significantly inhibit cell migration and cell growth through targeting of soluble collagens. These results suggest that let-7g may suppress HCC metastasis through targeting collagen and that let-7g could be used as a tool to predict poor survival.

Given the predominant underlying fibrotic and cirrhotic conditions of the liver in those individuals prone to HCC and its recurrence, alterations of components of the inflammatory milieu have been suggested as factors which propel the formation and advancement of HCC. In particular, the activity of hepatic stellate cells (HSC), key features of fibrosis and cirrhosis, have been suggested as contributors to the HCC-prone microenvironment. A HSC-specific gene expression signature among tissue specimens of 319 HCC patients was recently identified and validated that is significantly and independently associated with HCC recurrence and survival [162]. Further computational analyses and immunohistochemical validation in a cohort of 143 HCC patients showed that the majority of alterations in patients with poor prognosis defined by HSC status were associated with peritumoral, rather than tumoral tissues. Furthermore, coculture studies demonstrate that HSCs preferentially affect monocyte populations, particularly CD14+ cells, within the microenvironment, that are related to a Th2-cytokine promoting shift in their inflammatory state. The interactions between HSCs and monocytes induce protumorigenic and progressive features of HCC cells by enhancing cell proliferation, migration, and tumor sphere formation. In sum, these results show that HSCs play a significant role in promoting HCC progression via interaction with and alteration of monocyte activities within the liver microenvironment.

Another hepatic stellate cell signature was recently identified in hepatitis C patients and was validated retrospectively in HCC patients to identify those with poor prognosis [163]. Thus, disrupting the interactions and signaling events between the inflammatory milieu and components of the microenvironment may be useful therapeutic strategies for preventing HCC tumor relapse. In addition, Tao et al. analyzed hepatocytes isolated from HBV-HCC cases on a 27 K array and identified hypermethylated genes. Overall, these studies highlight the significant role of the field cancerization effect to initiate and drive cancer progression [164]. More recently, other factors, such as the diet and the microbiome, are being studied to determine their roles in influencing the liver microenvironment [165].

5.5 Tumor Heterogeneity and Subclassification

Tumor heterogeneity may result from different cells of origin, range in patient ethnicity, etiology, underlying disease, and diversity of genomic and epigenomic changes which drive tumor development. Molecular differences between tumors from different patients, intertumor heterogeneity, and between different areas of an individual tumor, intratumor heterogeneity, have been recognized, possibly emanating from the presence of cancer stem cells or selection by clonal evolution. Cancer genomic heterogeneity thereby results in varying degrees of clinical presentation and tumor biology, which impedes treatment options and poses a significant challenge to cancer management [166]. An emerging challenge in HCC clinical management is intratumor heterogeneity, whereby distinct cell populations within a given tumor may result in poor response or resistance to therapy [167]. Some initial attempts have been made to characterize the extent of intratumor heterogeneity in HCC. In a recent study of 120 tumor areas from 23 HCC, intratumor heterogeneity measured by morphology, imunohistochemistry, and/or gene mutation status was found in the majority of specimens [168]. A comprehensive omics approach geared toward this feature of tumor biology is necessary for improving HCC clinical management. Findings of this type indicate that single tumor biopsies and the data collected from such specimens may not provide the entire portrait of alterations occurring in a given tumor. This nonuniformity of molecular changes currently represents a significant challenge in the development of targeted therapy for HCC.

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 either related to IFN-associated inflammation or apoptosis while another cDNA study composed of 19 HCC cell lines, found two subtypes that were correlated with AFP expression [169, 170]. 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 [171]. A separate cDNA study of HCCs from 10 patients found several genes related to histological subtype [172]. In an OLIGO study of well-differentiated HCC versus hepatocellular ademonas, 63 genes were found to be differentially expressed, demonstrating molecular differences despite similarities in morphology [173]. Another OLIGO study identified 31 genes that differed between early and advanced HCV-HCCs [174]. 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 [175, 176]. TMA has also been applied to identify tumor subgroups. Recently, Tan et al. applied comparative proteomics to HCC tumor tissues and identified a three-protein panel (HSP70, ASS1, and UGP2) that could stratify HCC patients into two groups [145]. A miRNA-based classification of three subclasses of HCC has also recently been proposed [177]. Among the proliferation class, miR-517a is an oncogenic miRNA that promotes tumor progression. Thus, there is a rationale for developing therapies that target miR-517a for patients with HCC.

We recently hypothesized that AFP^+ and AFP^- HCC tumors differ biologically. Using global microRNA profiling, we found that miR-29 family members were significantly downregulated in AFP^+ tumors with a significant inverse correlation between miR-29 and DNMT3A gene expression [178]. We also showed that AFP^+ and AFP^- HCC tumors have distinct global DNA methylation patterns, with an increased DNA methylation in AFP^+ HCC. AFP expression induces protumorigenic features along with miR-29a inhibition and DNMT3A induction. AFP also inhibited transcription of the miR-29a/b-1 locus via c-MYC binding to the miR-29a/b-1 transcript. Further, AFP expression promotes tumor growth of AFP^- HCC cells in nude mice. Thus, tumor biology differs considerably between AFP^+ HCC and AFP^- HCC and that AFP is a functional antagonist of miR-29, which may contribute to global epigenetic alterations and poor prognosis in HCC.

Recent attempts have been made to utilize profiling data to molecularly classify HCC in order to identify common homogenous subgroups of this disease which may respond more preferably to certain types of treatment. Studies indicate that aberrant activation of signaling pathways involved in cellular proliferation (e.g., epidermal growth factor and RAS/mitogen-activated protein kinase pathways), survival (e.g., Akt/mechanistic target of rapamycin pathway),

differentiation (e.g., Wnt and Hedgehog pathways), and angiogenesis (e.g., vascular endothelial growth factor and platelet-derived growth factor) are present in particular groups of HCC tumors [179, 180]. These cancer genes are thus ideal targets for biotherapies, underscoring the importance of tumor biology to medicine.

5.6 Stem Cell-Based Signatures

The heterogeneic nature of HCC and variability of its prognosis suggests 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 [52]. 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. cDNA arrays were used to identify a HCC subtype with features of hepatic stem cells that expresses AFP and a cell surface hepatic stem cell marker, EpCAM [56, 181]. EpCAM-positive cells from this subtype have self-renewal and differentiation traits and can initiate highly invasive HCC in NOD/SCID mice [182]. 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.

It was 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 [183]. A global microRNA microarray approach was used to explore whether certain microRNAs were associated with HCC stem cells. It was found that the conserved microRNA-181 family members were upregulated in HCC stem cells. Inhibition of microRNA-181 led to a reduction in number and tumor initiating activity of HCC stem cells while addition of microRNA-181 led to an enrichment of this cell type. In further studies, microRNA-181 could directly target transcriptional regulators of differentiation in the liver and an inhibitor of Wnt-beta-catenin signaling. In addition, Wnt/beta-catenin signaling transcriptionally activates microRNA-181s in HCC [184]. These results suggest a novel regulatory link between microRNA-181 family members, Wnt/beta-catenin signaling, and liver cancer stem cells and implies that molecular targeting of microRNA-181 or Wnt/beta-catenin signaling may eradicate hepatocellular carcinoma (HCC).

Studies have also recently explored whether specific microRNAs exist in hepatic cancer stem cells (CSCs) that are not expressed in normal hepatic stem cells by assessing the microRNA transcriptome of HCC specimens by small RNA deep sequencing [185]. It was found that miR-150, miR-155, and miR-223 were preferentially highly expressed in EpCAM+ HCC cells and their gene surrogates were associated with patient prognosis. Further studies showed that suppressing miR-155 resulted in reduction of EpCAM + HCC cells, reduced HCC tumorigenicity, and shortened overall survival and time to recurrence of HCC patients. Thus, miR-155 was highly elevated in EpCAM1 HCC cells and might serve as a molecular target to eradicate the EpCAM+ CSC population in human HCCs.

While EpCAM seems to be a positive marker of HCC CSCs, others have shown that HCC cells may also be positive for CD133 or CD90, indicating that these antigens are also features of cancer stem cells [186, 187]. 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 dedifferentiation 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.

5.7 Future Directions

5.7.1 Sequencing

Recently, a more comprehensive view of the genome has been made through the use of sequencing technology. We are now able to define specific mutations in the protein-coding region (exome), the whole genome, and various RNA transcripts. These approaches have led to the

Table 5.1 A summary of HCC DNA sequencing studies

Platform*	Sample size	Candidate driver genes	Study/year	References
Whole genome	147	ATM, CTNNB1, ARID1A, IGSF10, TP53,	Fujimoto et al. (2012)	[218]
Whole genome	88	CTNNBI, LRP1B TP53	Kan et al. (2013)	[22]
Whole genome	608	CTNNB1, TERT, TP53	Totoki et al. (2014)	[193]
Whole exome	149	ARID1A, AXIN, CTNNB1, RPS6KA3, TP53	Guichard et al. (2012)	[219]
Whole exome	11	TERT	Woo et al. (2014)	[190]
Whole exome	110	ARID1A, TP53	Huang et al. (2012)	[191]
Whole exome	87	CTNNB1, TP53	Cleary et al. (2013)	[188]
Whole exome	235	ALB, ARID1A, AXIN1, CTNNB1, TERT, TP53	Schulze et al. (2015)	[194]

*Manuscripts were selected based on the use of next-generation sequencing methods in human cohorts. Candidate driver genes are presented in alphabetical order and represent those genes found at greater than 10 % frequency in the noted study

discovery of novel genes in HCC. For example, whole exome sequencing has identified alterations of ARID1A, RPS6KA3, IRF2, NFE2L2-KEAP1, KMT2A in HCC [20, 188]. In addition, ARID2 has been implicated in HCV-associated HCC by whole exome sequencing, while CTNNB1 was found to have a pivotal role in HBV-HCC [189, 190]. This method has also been used to identify important genes associated with HCC metastasis, including CUL9, FGD6, KDM6A, AKAP4, and RNF139 [191].

The identification of genomic alterations in the full genome has also been attempted to understand the alterations occurring in noncoding regions and by structural rearrangements of the genome (Table 5.1). Several thousand somatic mutations and numerous chromosomal alterations were found by whole genome sequencing of a single HCV-HCC case by Totoki et al. In a study of mainly HBV-HCC, the JAK/STAT and WNT/Bcatenin pathways were found to be important drivers [192]. Recently, this work has been expanded in over 500 liver cancer cases, uncovering 30 candidate driver genes and 11 core pathways including metabolic enzymes, chromatin remodelers, and TERT as a central and ancestry-independent node in HCC [193]. In addition, DNA mismatch repair genes and chromatin regulators, including ARID1A, ARID2, and MLL3 were mutated in a study of HCC including both HBV and HCV patients [21]. In an exome sequencing study by Schulze et al., TERT promoter mutations were identified as early events in HCC, while TP53, CTNNB1, CDKN2A and FGF family members were related to more advanced HCC stages [194]. Whole genome sequencing has also allowed for the identification of viral integration sites caused by the DNA virus, HBV, and genomic aberrations that occur near those sites. Important integration sites include TERT, MLL4, FN1, and CCNE1 [22, 195]. Retrotransposon insertions and repetitive sequences have also been explored by whole genome sequencing. Two long interspersed nuclear element-mediated somatic changes in MCC and ST18 have recently been described in HCC [196].

RNA sequencing, meanwhile, provides an extension of transcriptomic profiling by allowing for the assessment of translocation and inversions of transcripts, noncoding RNAs, and splicing events. Splicing variants for several genes have been reported in HCC including TCF4, KLF6, p73, and LLGL1 [197]. RNA editing events have also been explored by this methodology and have identified a gain of function activity in the AZIN1 gene in HCC along with RNA editing roles of BLCAP [198–200]. These studies are rather small in sample number and await further exploration in larger datasets.

5.7.2 Circulating Tumor Cells

Although hepatic resection and liver transplantation are the main modalities of curative HCC treatment, approximately 40 % of hepatectomy patients and 10 % of transplant patients develop postoperative recurrences. One factor that is thought to underlie this outcome is the presence of circulating tumor cells (CTCs) which may be released from the primary tumor or metastatic lesions. In the last decade, effort has been placed on identifying and improving technology and methods to detect CTCs, understand their role in tumor biology and usefulness as tumor biomarkers. These include enrichment methods based on physical characteristics and/or immunological markers, microfilters, density gradient centrifugation, and microfluidic chips [201, 202]. Once enriched and isolated, various methods are used to characterize CTCs including nucleic acid analysis, cytometric analysis, and functional analysis. The characterization and enumeration of CTCs may be a significant advance in our understanding of tumor heterogeneity, patient stratification for treatment or treatment response, and risk of relapse.

Table 5.2 A summary of HCC integrated omics studies

Integrated platforms*	Sample size	Candidates/signatures	Study/year	References
Double platform integration				
Transcriptome + Metabolomics	356	SCD1 (lipid signature)	Budhu et al. (2013)	[210]
Transcriptome + aCGH	61	Metastasis genes	Roessler et al. (2015)	[220]
Transcriptome + aCGH	76	PROSC, SH2D4A, and SORBS3 (tumor suppressors)	Roessler et al. (2012)	[207]
Transcriptome + aCGH	380	YY1AP1 (metastasis/stem cell)	Zhao et al. (2015)	[208]
miRNA + mRNA	100	miR-148-ACVR1/BMP	Li et al. (2015)	[209]
RNA Seq + DNA Seq	2	BLCAP (RNA editing)	Hu et al. (2015)	[199]
Methylation + Transcriptome	71	SMPD3, NEFH (tumor suppressors)	Revill et al. (2013)	[213]
Methylation + Transcriptome	128	CFH, MYRIP, PSRC1, MRE11A and MYO1E (tumor recurrence)	Yang et al. (2011)	[214]
Triple platform integration				
RNA Seq + DNA Seq + SNP	174	TTK (mitotic checkpoint)	Miao et al. (2014)	[217]
Methylation + Transcriptome + aCGH	63	PER3, IGFALS, protein Z (tumor suppressors)	Neumann et al. (2012)	[216]
Methylation + Transcriptome + SNP array	49	COL1A1 (survival)	Hayashi et al. (2014)	[215]

*Manuscripts were selected based on the integration of two or more omic platforms and the use of human cohorts

A few studies have been published regarding CTC detection and characterization in HCC. The clinical usefulness of CTC counts was reported in a preliminary study by Vona et al. in 44 HCC patients showing association of CTCs with later disease stage and shorter survival [203]. Detection of CD45(−)CD90(+)CD44(+) or EpCAM(+) cells have also been employed to predict HCC recurrence and metastasis [204–206]. Current strategies are focused on further characterizing CTCs and understanding their modes of release and circulation in order to prevent or reduce the risk of recurrence, metastasis, and improve survival rates.

Our ability to define specific CTCs by single markers or overlap of specific markers will also aid in understanding the pools of CTCs that may be present in a given tumor or tumor subtype that could allow us to better identify and stratify HCC patients for effective treatment, etc. This may also lead to strategies for targeting and/or eliminating CTCs in order to prolong patient survival. Although the amount of data and evidence concerning CTCs are growing in the HCC field, currently there is still a lack of definitive evidence that the detected cells are specific to HCC, capable of stem-like abilities and initiate metastasis or recurrence. In addition, current CTC capture techniques will need to be improved in order to increase the purity of isolated cells and their yield. Overall, CTCs represent an important new strategy to identify markers for patient relapse and poor survival and may be targetable populations to reduce these outcomes.

5.7.3 Data Integration

While array-based technologies have allowed us to define molecular alterations at various levels of the genome, it is important to note that these factors do not act on their own, but rather, make up complex networks that span several levels of genomic and genetic signaling. In this vein, it is important for us to be able to understand how these factors interact and/or are affected by one another to produce the final phenotype that is observed. Thus, many researchers involved in high-throughput genomics have begun to explore signaling networks, rather than single molecules, as methods of defining important molecular nodes and drivers of HCC. Such integrated approaches are thought to be an improved strategy of resolving the important and key molecules that cause HCC and allow it to progress (Table 5.2).

We have also recently used integrative approaches to identify HCC driver genes. For example, we have combined high-resolution, array-based comparative genomic hybridization, and transcriptome analysis of HCC samples to identify and validate a 10-gene signature associated with chromosome 8p loss and poor outcome [207]. Functional studies demonstrated that three gene products among the 10-gene signature have tumor suppressive properties. Integrated genomics has also recently been used to identify YY1AP1 as an oncogenic driver in stem-like HCC [208]. In

an integration study of miRNA and mRNA profiles, the miR148a-ACVR1/BMP circuit was useful in defining a stem cell-like aggressive subtype of HCC [209]. Metabolite and mRNA profiles have also been integrated to define key signaling events that can alter the fitness of EpCAM+ AFP + HCC cancer stem cells [210]. Our analysis revealed tumor-specific and stem cell-like-specific metabolites linked to patient survival along with correlating significant genes in the stem cell-like tumor subgroup. In particular, stearoyl CoA desaturase (SCD), a key enzyme involved in fatty acid biosynthesis, and its related metabolites were highly elevated in stem cell-like HCC and are associated with HCC survival and may functionally contribute to HCC stemness and aggressiveness. We have also recently compared and contrasted global metabolic profiles between liver, breast, and pancreatic cancer tissues and found that metabolites are principally unique to each tissue and cancer type. Thus, metabolic profiling could be applied as cancer classification tools to differentiate tumors based on tissue of origin [211].

To aid in the integration of multiple omics data, we have proposed an integrative subgraph mining approach, called iSubgraph to discover patterns of miRNA-gene networks which could be used for patient stratification in HCC [212]. This algorithm could detect cooperative regulation of miRNAs and genes with highly stable class predictions. The HCC subgroups identified by the algorithm have different survival characteristics with key roles of specific genes in HCC subgroups. Thus, our method can integrate various omics data derived from different platforms and with different dynamic scales to better define molecular tumor subtypes.

Integrative genomic analysis of genome-wide methylation and gene expression data identified possible key targets in HCC. Recently, using this method, the tumor suppressive roles of SMPD3 and NEFH have been demonstrated in HCC [213]. Evidence was provided that SMPD3 is a potent tumor suppressor gene that could affect tumor aggressiveness, while a reduced level of SMPD3 is an independent prognostic factor for early recurrence of HCC. This method was also used to identify genes associated with HCC recurrence, including CFH, MYRIP, PSRC1, MRE11A, and MYO1E [214]. Triple-combination array analysis of expression arrays, SNP array, and methylation array successfully identified COL1A1 as a candidate survival-related gene in HCCs. Epigenetic downregulation of COL1A1 mRNA expression might have a role as a prognostic biomarker of HCC [215]. A combination of genome-wide methylation, array CGH, and gene expression was also used to identify PER3, IGFALS, protein Z as HCC tumor suppressors [216]. Whole genome sequencing has been integrated with transcriptome sequencing and SNP genotyping to identify a dual-specificity protein kinase, TTK as a prognostic indicator of HCC [217].

Integration among various levels of omics signaling may help to further define the key players that promote HCC and affect its progression. For clinical application, it is also useful to integrate omic information with current clinical triage methods, including tumor staging and pathology, to further refine patients into risk groups. This combined information can then be applied to stratify patients for the most appropriate and likely to be most effective treatment regimens. This strategy underlies the topic of precision medicine, whereby a more individualistic approach based on the combination of science and medicine is used to manage patient care.

5.8 Summary

The advent of array technology has provided a high-throughput methodology to assess the genome-wide changes that occur during hepatocarcinogenesis and its progression. 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.

Arrays 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 arrays, 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 technique such as laser capture microdissection and automation has 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 posttranscriptionally. Therefore, elements such as protein localization and modification are important elements to be included in HCC profiling. Difficulties in data comparison and integration must also be addressed which ensues from the use of multiple array 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 array statistical analysis and reporting such as those established by the International Microarrays Gene Expression

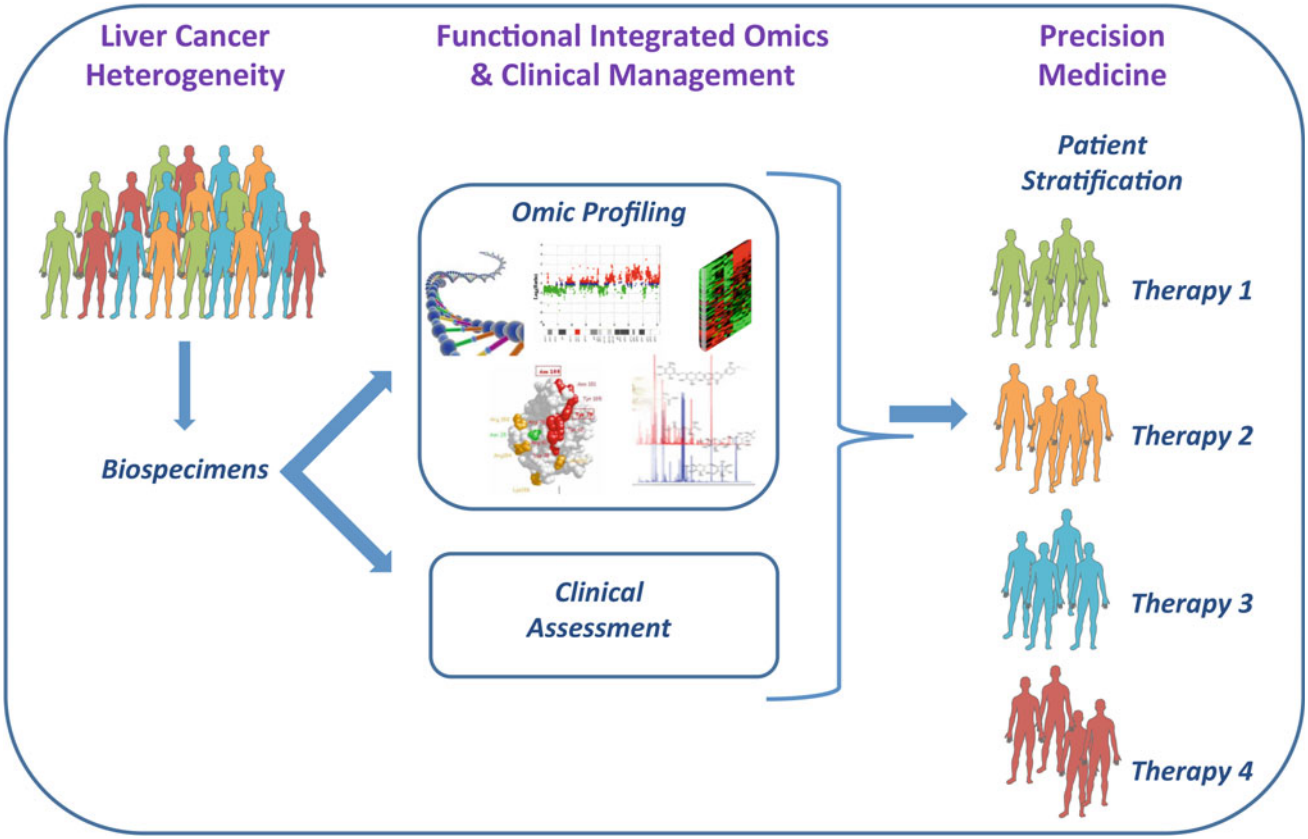

Fig. 5.1 Functional and integrated omic profiling for biomarker identification, validation, and clinical utility. Widescreen genomic profiling of hepatocellular carcinoma (HCC) has identified multiple biomarkers on the gene, protein, and genomic scale. These biomarkers are useful for understanding HCC biology and clinical application. The mechanistic and clinical information gleaned from genomic profiling studies can be combined using computational strategies to identify promising novel therapeutic markers for diagnosis, treatment, and prognosis of HCC. Such methods will allow progression toward precision medicine encompassing new and selective therapeutics and preventative therapy

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. Lastly, each array can only provide information concerning the targets that are included in that array. Thus, integrative analysis of multiple platforms may be required 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 also be required to make such assessments. Recently, systems have been developed 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 a cost-effective and accurate manner and may allow for ease in cross-platform-type analyses since an enormous multilevel dataset can be achieved with a relatively small amount of the same starting material. Overall, integrating global molecular profiling data along with mechanistic/functional studies may improve the diagnosis, treatment, and prognosis of HCC patients.

Although multiple publications have identified and validated diagnostic and/or prognostic HCC markers, 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 noninvasive 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 (Fig. 5.1). The HCC-related genomic expression studies presented in this chapter along with future studies and advances in array 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 patients. We are now at the brink of clinically implementing biomarkers identified from global array profiling to improve HCC diagnosis, treatment, and outcome.

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