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Clinicopathologic Risk Factors and Biomarkers Associated with Recurrence in Patients after Curative Resection of Hepatocellular Carcinoma

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ABSTRACT

Liver cancer, primarily hepatocellular carcinoma **(HCC)**, is the third leading cause of cancer-related death worldwide, and the ninth leading cause of cancer-related death in the United States. Surgical resection is an important curative treatment for hepatocellular carcinoma **(HCC)**, but the prognosis following surgery differs substantially and such large variations are mainly unexplained. In this chapter, we have reviewed a number of clinicopathological parameters associated with HCC prognosis. The molecular pathogenesis is extremely complex and heterogeneous, and molecular information has not yet impacted treatment decisions. Deregulation of signaling pathways and cellular processes have been implicated in cancer recurrence; enhanced epithelial-mesenchymal transition **(EMT)** and angiogenesis from the activation of TGF-β, WNT signaling, and HGF/c-met signaling is found to predict cancer recurrence and poor patient outcome. Immune evasion from

abnormal secretion of cytokines, chemokines, and impaired immune checkpoints in the immune microenvironment around the tumor is emerging as an important cellular process for cancer recurrence. Furthermore, data have shown that oxidative stress may be a hallmark of cancer aggression and risk of recurrence. Finally, microRNAs have been found to orchestrate the processes of metastasis. We hope that a review of the possible mechanisms of cancer recurrence will help the reader gain insight into the clinical and molecular mechanisms underlying HCC recurrence, and identify possible therapeutic targets to prevent or lower the risk of this recurrence.

INTRODUCTION

The implementation of successful hepatocellular carcinoma **(HCC)** screening programs and high quality imaging, combined with improvements in perioperative medical and surgical management, has made hepatic resection a preferred treatment modality for hepatocellular carcinoma **(HCC)** in selected patients. Both the American Association for the Study of Liver Disease **(AASLD)** [1] and the European Association for Study of the Liver **(EASL)** [2] have defined criteria for potential resection candidates using the Barcelona Clinic Liver Cancer **(BCLC)** classification [3]. Ideal patients are BCLC Class 0, defined as a having a single tumor < 2 cm in diameter without vascular invasion/satellites, good performance status **(ECOG - 0)**, and well-preserved liver function (Child-Pugh A). Although liver transplantation remains the favored surgical modality, owing to lower risk of recurrence and improved long-term survival due to removal of the cirrhotic liver, its use is not without limitations. Risks associated with hepatic decompensation and neoplastic progression while awaiting transplant, as well as lifelong immunosuppression post-transplant increase morbidity and mortality. Furthermore, the constraint of a limited supply of organs increases the appeal of surgical resection of the tumor when feasible. As surgical experience and perioperative care have improved, death rates after liver resection for HCC have approached zero in experienced centers [4]. Despite excellent immediate postoperative outcomes, the longer-term risk of tumor recurrence cannot be ignored. The 5 year survival rate is estimated to be between 30-60% [5], while the risk of tumor recurrence is estimated to range from 75-100% at 5 years [6]. Attention has therefore been given to improving long term outcomes for patients undergoing HCC resection by defining and addressing risk factors associated with HCC recurrence.

In this chapter we will first review the associated clinical features of tumors, before addressing surgical techniques and host factors. A discussion of emerging molecular biomarkers and their role in the prediction of clinical outcome of patients after curative resection of hepatocellular carcinoma will ensue.

Conventional Risk Factors that Predict Cancer Recurrence

HCC recurrence can be divided into early and late phases. The timing of tumor recurrence reflects the oncogenic mechanisms and thus potential for therapies. Early recurrence occurs within two years of liver resection and is thought to be related to micrometastasis already present at the time of surgery. In contrast late recurrence occurs ≥ 2 years after initial resection and is attributed to de novo tumor formation [7].

While timing and pathophysiological mechanisms vary between early- and late- recurrence groups, the clinical risk factors for recurring malignancy can be considered universal, and can be categorized into tumor, host, and surgical factors.

Vascular invasion - a strong predictor for HCC recurrence

Conventional pathologic tumor characteristics have been extensively studied and are among the strongest predictors of HCC recurrence after resection. Among these characteristics, vascular invasion has been confirmed as one of the most consistent predictors for tumor recurrence and early death [8,9]. Macrovascular invasion can be detected radiographically or by gross inspection at surgery and is strongly associated with a poor prognosis. In a review of 384 patients undergoing HCC resection, Roayaie et al. demonstrated that the median time to recurrence in patients with macrovascular invasion was 6.7 months compared with approximately 30 months for those free of vascular involvement [10]. In contrast, microvascular invasion **(MVI)** is detected in resected specimens only and is defined by the presence of tumor emboli within the central hepatic or portal veins or the large capsular vessels. Owing to wide inter-/intra-observer variability, as well as lack of definition and grading of MVI, there is significant heterogeneity regarding impact on survival. Nonetheless, in a recent systematic review the relative risk of MVI on 3-year and 5-year disease-free survival **(DFS)** was 1.82 [1.61-2.07] and 1.51 [1.29-1.77], respectively [11]. This finding was confirmed by another investigation which reported that MVI predicted a 1.4 fold decrease in time-to-recurrence [8]. Thus it can be concluded that MVI positively correlates with disease recurrence. Indeed, the predictive value of MVI is so well recognized it has been used as an independent prognostic indicator for the risk of recurrence. For example, a direct relationship has been established between the size of the primary lesion and likelihood of MVI; primary nodules greater than 4 cm diameter have been shown to increase the risk of recurrence [12,13], with a direct correlation at > 10 cm. Satellite nodules around the main tumor, absolute number of tumors, and pathologic tumor-node-metastasis **(pTNM)** staging are also established predictors of MVI [14].

Tumor differentiation- a possible predictor

Risk associated with tumor differentiation is less clear, due in part to the challenge arising from the close-knit association between poorly differentiated tumors and vascular invasion. However, several recent studies have demonstrated an increase in recurrence of poorly differentiated HCCs [15], particularly in patients with early (< 2 years) recurrence [16]. Prognosis related to tumor encapsulation has been similarly conflicting. A recent study by Wu et al. showed tumor encapsulation to be prognostic of improved DFS and overall survival **(OS)**, but only in lesions > 5 cm [17]. Similar findings have been shown by others [18,19], whereas Adachi et al. showed the presence of a capsule to be a risk factor for portal venous invasion, predicting that capsule vessels had been invaded by malignant cells [20].

Alpha fetoprotein (AFP)

Biomarkers such as alpha fetoprotein **(AFP)** and lens culinaris agglutinin-reactive fraction **(AFP-L3)** have also been shown to be predictive. Of these, AFP is best studied, but mostly in the prediction of recurrence in the transplant setting. Although less data exist post-hepatectomy, this biomarker does seem to have prognostic value [21]. However, regular application is limited by the wide variety of cutoff values used in studies: values range from ≥ 32 ng/mL [7] to > 1000 ng/mL [21]. Despite this variation, it is generally accepted that an AFP value >1000 ng/mL represents a high risk for recurrence. AFP-L3 is proposed to be a more specific marker of HCC disease status, particularly in patients with background cirrhosis or hepatitis. Zhang et al. reviewed 395 patients undergoing liver resection, and examined the use of AFP-L3 in prognosis and surveillance of outcome [22]. A positive preoperative AFP-L3, defined as ≥10% of total AFP or continuously positive or negative-turn-positive AFP-L3, were predictive of more aggressive tumor behavior, higher tumor recurrence, and poorer clinical outcomes. Despite this, AFP-L3 and other markers, such as des-gamma-carboxy prothrombin **(DCP)** and protein induced by vitamin K absence or antagonist II **(PIVKA-II)**, have not gained wide acceptance in general clinical practice.

Surgical factors

Laparoscopy has gained wide acceptance across many surgical indications. Laparoscopic HCC resection was first reported in the late 1990s, and a sizeable body of literature exists to show its efficacy. A meta-analysis of 494 patients showed there was no difference in oncologic outcomes, OS, or DFS rates. Furthermore, perioperative outcomes including blood transfusion requirement, postoperative morbidity, and length of hospital stay were superior following laparoscopy compared to conventional open operation [23]. Long-term survival following laparoscopic resection is also shown to be equivalent, and even trend toward being significantly beneficial, to conventional open operation, as shown in a meta-analysis by Cheung et al. where DFS was 78.5 months versus 29 months (P = 0.086) and OS was 92 months versus 71 months (P = 0.142) [23].

Data regarding the impact of tumor-free margins on recurrence are varied and conflicting. This may be due to differences in margin versus non-margin intrahepatic tumor recurrence. Because most recurrences result from hematogenous spread, it is understandable that tumor-free margins have little effect on intrahepatic tumor recurrence. Nonetheless, many surgeons advocate for a tumor-free surgical margin of at least 1 cm [24], while other data suggest that wider margins can lead to a significant reduction in recurrence. In a randomized controlled trial, Shi et al showed that a margin of 2 cm decreased recurrence and improved survival rates [25], while others have recommended margins of 2-3 cm even for small tumors [26]. Despite these findings other studies have been unable to demonstrate any significant effect of resection margin size on recurrence [27].

Another aspect of surgical technique is anatomic versus non-anatomic resection. Anatomic resection **(AR)** is defined by removal of an entire hepatic segment and its associated vascular

supply. Due to previously proposed pathogenic mechanisms relating to vascular invasion, the theoretical benefit in removing segmental vascular supply to decrease recurrence risk becomes clear. In contrast, sparing liver parenchyma through non-anatomic resection **(NAR)** may be important in decreasing the risk for post-operative liver failure in cirrhotic patients. Unfortunately, as it relates to recurrence risk, there are no current randomized controlled trials examining AR versus NAR. The largest retrospective study that examined 5781 patients who underwent liver resection demonstrated an DFS benefit of AR over NAR [27]. More recently, Cucchetti et al, performed a meta-regression analysis of over 9000 HCC patients, finding that AR was associated with improvement in both DFS and OS [28]. Notably, NAR patients had a higher prevalence of cirrhosis, more advanced hepatic dysfunction, but smaller tumor sizes, leading to the conclusion that these findings may be due, in part, to the impact of liver function on survival.

One additional, non-technical factor relates to intraoperative blood loss and perioperative transfusion. Multiple studies have confirmed an increased risk of HCC recurrence related to both intraoperative blood loss [29] and perioperative transfusion [30]. It is thought that transfusion promotes recurrence through suppression of host antitumor immune response. Thus, in order to mitigate recurrence risk, surgical technique should be meticulous, paying particular attention to minimizing blood loss and transfusion.

Host factors

Generally, patient demographics such as age and gender are not considered prognostic. However one study showed better outcomes for women than men when undergoing the same type of resection; women had better survival rates and a lower rate of recurrence. This finding was attributed to less aggressive pathologic tumor characteristics, including encapsulation and lower tumor invasiveness [31]. Interestingly, the underlying cause of HCC, such as alcohol or hepatitis B or C viral infections, seems to have little bearing on early (< 2 years) tumor recurrence. This phenomenon is likely related to micro-metastatic mechanisms occurring during this time frame, for which underlying etiology would have less importance. In contrast, in cases of late (≥ 2 years) recurrence, some authors suggest an increased risk with hepatitis C compared to hepatitis B viral infections [32,33]. Other studies have shown an increased recurrence risk associated with alcohol abuse [34]. These studies indicate that fundamental tumor characteristics (e.g., multifocality or grade and type of underlying hepatitis infection) and the etiology of liver disease are independent risk factors.

Inflammatory activity has been consistently shown to be predictive for HCC [35, 36]. Untreated viral hepatitis is a persistent cause of inflammation after resection, which accordingly poses an increased risk for recurrent HCC [32]. Thus, not surprisingly, the treatment of both HBV and HCV with antiviral therapy has been shown to decrease HCC recurrence [37,38]. The correlation between the degree of liver fibrosis or cirrhosis and HCC recurrence is more controversial. Imamura et al showed that the stage of fibrosis was prognostic in patients without cirrhosis but

not in patients with cirrhosis [7]. Some studies have described a 2.4 fold increased risk of HCC recurrence in cirrhotic patients [14], while others have shown no association at all [39,40].

HCC is known as an inflammation-associated cancer [41,42]. Biomarkers of chronic inflammation have been suggestive in the development and recurrence of HCC. It is known that chronic inflammation leads to oxidative/nitrosative stress and lipid peroxidation **(LPO)**, generating excess reactive oxygen species **(ROS)** and reactive nitrogen species **(RNS)** together with aldehydes, which can react with DNA bases to form promutagenic DNA adducts through either endogenous or exogenous insults [43]. Oxidative stress has emerged as an important player in the development and progression of liver carcinogenesis related to different pathological conditions (e.g. HBV- and HCV- induced liver disease) [44]. It has been known that the HCC incidences in the USA are largely associated with HCV-associated cirrhosis, but changes in the incidences of HCC observed in epidemiological studies have shown that obesity and diabetes are also risk factors for HCC [45]. Both of these factors are also known to associate with increased oxidative stress [46,47]. This notion is supported by a large number of chemopreventive epidemiological studies [48, 49]. Thus, knocking out antioxidant defenses increases the rate of cancer. For example, knockout mice lacking CuZuSOD (copper-zinc superoxide dismutase) are found to have increased liver carcinogenesis [50]. Another mouse model showed that knocking out nuclear respiratory factor-1 **(Nrf1)**, which is an essential transcription factor for mediating oxidative stress, induces steatosis, fibrosis, and eventually liver cancer [51]. A major oxidative stress and promutagenic DNA adduct marker, 8-oxo-7, 8-dihydro-2'-deoxyguanosine (8-oxo-dG), was found to be increased during hepatocarcinogenesis which suggests a role of oxidative stress in HCC formation [52, 53]. The concept of oxidative stress playing a role in carcinogenesis of the liver is also supported by data from a multicenter study, which used tissue microarrays screening. Cytochrome P450 1A2 **(CYP1A2)** in non-cancerous tissue is found as the predictive factor for HCC recurrence [54].

Serum quantification of reactive oxygen metabolite **(d-ROM)** derivative levels, a simple method for measuring hydrogen peroxide, is found to predict the risk of HCC recurrence after surgical resection or radiofrequency ablation **(RFA)** [55]. In a HCV/HCC clinical trial, 8-oxo-dG (oxidative DNA lesions) and hydroxynonenal **(HNE)** protein adducts (lipid peroxidation **[LPO]** protein lesions) are found to support the hypothesis that HCV-induced inflammation causes oxidative DNA damage and promotes hepatocarcinogenesis; greater oxidative stress led to a higher incidence of HCC recurrence. Furthermore, levels of γ-hydroxy-1, N2-propanodeoxyguanosine **(γ-OHPdG)**, a product of acrolein that is generated by endogenous LPO [56], are associated with greater incidence of HCC recurrence post resection (unpublished data).

EMERGING PREDICTORS FOR CANCER RECURRENCE

Deregulated pathways and genes in HCC that predict cancer recurrence

Using integration of genomic, transcriptomic and proteomic information, numerous pathways are shown to be deregulated in human HCC, including growth factors (e.g., epidermal growth

factor **[EGF]**, insulin-like growth factor **[IGF]**, and hepatocyte growth factor **[HGF]**); cytoplasmic intermediates (e.g., **AKT/MTOR** and **RAS/MAPK**); angiogenic factors (e.g., vascular endothelial growth factor **[VEGF]**, fibroblast growth factor **[FGF]**, and platelet derived growth factor **[PDGF]**); and factors involved with differentiation cascades **(WNT/β-catenin, Hedgehog** and **Notch)** [57-59]. Artificial activation of some of these pathways in experimental models has exhibited induction of liver tumors [60,61]. It has been suggested that activation of these pathways clinically predicts the aggressive behavior of HCC and poor clinical outcome of HCC patients. Particularly, HGF/MET, EGF, and WNT pathways have been evaluated as predictors of recurrence after curative resection of HCC. A C-MET-regulated expression signature defined a subset of HCC that showed worse prognoses [62]. C-Met protein levels in HCC have been found to predict the risk of cancer recurrence after curative resection of HCC. In 59 patients who had curable resection of their HCC, recurrence-free survival **(RFS)** was 166 days (95% CI: 99 - 232.9) for high c-MET expressing tumors vs. 748 days (95% CI: 355.2-1140.8) for low c-MET expressing tumor ($p < 0.0001$ [63]. Up-regulation of EGF in cirrhotic-adjacent tissue was also found to be predictive of poor prognosis in patients with HCC treated with surgical resection [64]. More recently the single-nucleotide polymorphism of EGF was shown to predict risk of recurrence after hepatectomy [65]. Furthermore, higher levels of RAC GTPase-activating protein 1 **(RACGAP1)** was associated higher recurrence of HCC after resection, with a hazard ratio of 2.71 (95% CI: 1.27-5.74, $p = 0.0096$) (20).

Tumor maintenance relies on neoangiogenesis, the formation of new blood vessels from preexisting vascular beds. Notably, high vascularization is a hallmark of human HCC [66]. Several preclinical studies have revealed VEGF and fibroblast growth factors **(FGFs)** as major drivers of proangiogenic signals. VEGF, considered one of the most potent angiogenic factors, is often overexpressed in HCC, as well as its preferred receptors, VEGFR1/2. Moreover, high VEGF expression levels have been consistently associated with more aggressive disease, and VEGF serum levels have been identified as a predictor of poor prognosis for resected HCC [67].

Transforming growth factor-β **(TGF-β)** signaling controls many cellular activities, including proliferation, migration, adhesion, differentiation, and modification of the cellular microenvironment. TGF-β binds to TGFβR1 and TGFβR2 receptor complexes and phosphorylates SMAD2 and SMAD3 that subsequently translocate SMAD4 to the nucleus, inducing transcription of target genes. The role of TGF-β in carcinogenesis is complex. It acts as a tumor suppressor in the early stages of tumor development by inhibiting proliferation and inducing apoptosis, but it also possesses oncogenic potential, which contributes to tumor progression later in carcinogenesis. Hernanda et al (40) reported a drastic elevation of nuclear SMAD4 localization in tumors of a subset of HCC patients. High expression of SMAD4 was further demonstrated to be functionally important for hepatoma formation and progression; simultaneous elevation of SMAD4 and p-SMAD2/3 in a subpopulation of HCC patients was significantly associated with poor outcome after surgery.

Programmed cell death exerts a key role in tissue development and homeostasis. Gai et al (38), provided the first evidence that P300/CBP-associated factor **(PCAF)**, a histone acetyltransferase **(HAT)**, acetylates cytoplasmic GLI1 protein and prevents its cytoplasmic-to-nuclear shuttling, which inhibits activation of Hh signaling, leading to a decrease in the Bcl-2/BAX ratio, and ultimately inducing cellular apoptosis. They also confirmed that HCC patients with high tumor PCAF expression had better postsurgical prognosis and were potentially more sensitive to 5-FU-based chemotherapy regimens. This is consistent with a previous study by Tuo et al who detected the expression of PCAF protein in 35 HCC patients from a medical center in China by IHC staining, and found that PCAF expression in HCC tissues was significantly associated with better long-term survival after surgery (39).

Alpha-L-fucosidase **(AFU)**, a liposomal enzyme present in all mammalian cells, that involved in the degradation of a variety of fucose-containing fucoglycoconjugates, has been proposed as a promising tumor marker in the diagnosis of HCC. In a study by Wang et al (42), a retrospective training data set and a prospective validation data set were used to evaluate the prognosis of HCC after partial hepatectomy. A total of 669 patients with histopathologically confirmed HCC were enrolled. Univariate and multivariate analyses were used to identify the prognostic significance of preoperative serum AFU. The investigators found that patients with a preoperative AFU (alpha-L-fucosidase) level >35 μl^{-1} had a lower RFS and OS than those with AFU levels ≤ 35 μl^{-1}. Patients with the higher AFU levels also had a greater tendency toward macrovascular invasion (43).

Four proteins-heat shock 70 kDa protein 1 **(HSP70)**, argininosuccinate synthase **(ASS)**, isoform 2 of UTP-glucose-1- phosphate uridylyltransferase, and transketolase-were shown to have the potential to differentiate metastatic relapse **(MR)** from nonrelapse **(NR)** HCC patients after validation by western blotting and immunohistochemical assays. The analysis revealed that a three-marker panel comprising HSP70, ASS1, and UDP-Glucose Pyrophosphorylase 2 **(UGP2)**, stratified the two groups of HCC patients (a statistically significant difference in levels of these three taken together was observed). This combination panel achieved high levels of sensitivity and specificity, which has the potential for clinical use in the identification of HCC tumors prone to MR [68] (44).

Even though current technology allows for DNA and RNA sequencing of HCC samples, due to the highly complex genetic abnormality in HCC, it remains a challenge to identify one or more HCC cancer hallmarks or abnormal pathways that will predict the risk of cancer recurrence in a patient. A different strategy is to identify a panel of genes or proteins that reflect the activation of multiple pathways that might predict recurrence after curative resection of HCC.

Development of gene signatures that predict HCC recurrence

The development and sophistication of high-throughput technologies will provide scientists with massive amounts of genomic information, enabling large integrative data efforts and identification of signaling 'hubs'. Transcriptomic methodology, such as DNA microarray allows

for the study of expression of all transcribed genes. DNA microarray uses a complementary DNA **(cDNA)** hybridized onto DNA probes that are present on a chip. This type of analysis is commonly used in HCC to identify predictive models. MicroRNAs **(miRNAs)** are small RNAs that are comprised of a 21-nucleotide sequence that serves as a building block for the future messenger RNA or DNA. MicroRNAs function as a regulator of gene expression together with transcription factors [69]. miRNA analysis is another testing tool used for the identification of predictive models in HCC. Proteomic studies are another major tool used to define predictive models in HCC; due to continuous protein changes within the tumor, transcription analysis often cannot demark disease status as well as proteomic analysis.

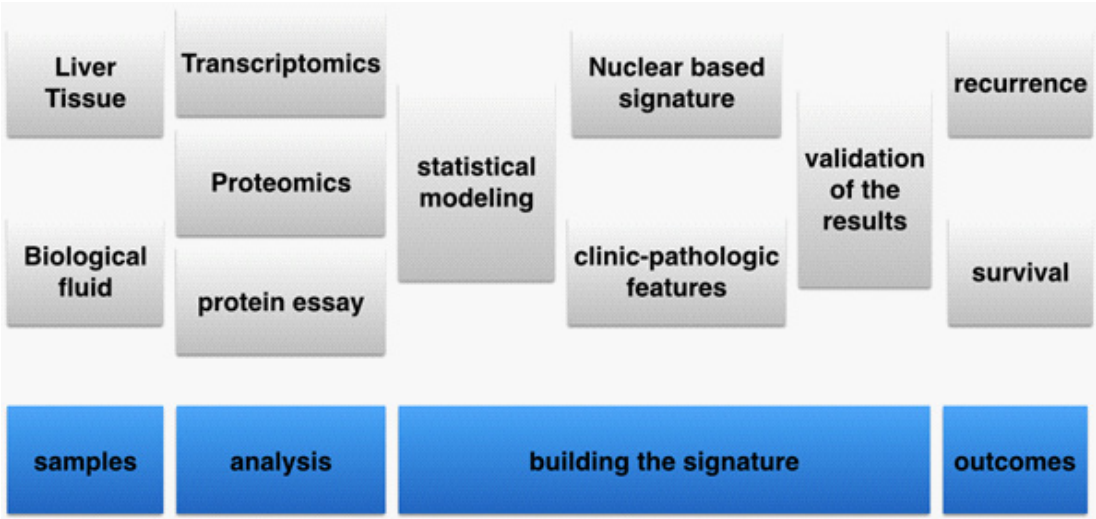

Figure 1: Systems used to predict outcomes of patients with hepatocellular carcinoma. Tissue samples (tumor and non-tumor) as well as biological fluids (blood, urine etc.) are used for molecular analysis via multiomics-based methodologies to obtain molecular based signatures for HCC recurrence. The combination of these signatures and clinicopathological features (e.g., HBV or HCV infection, serum AFP levels, and tumor staging) would be used to generate predictive systems for intrahepatic and de novo recurrence. Adapted from Lee et al, 2014 (6).

Molecular signatures in HCC

Many prognostic/predictive molecular signatures have been proposed but few have been validated and none have made it into common practice [70]. Hoshida et al. reported a statistically significant survival association with a 186-gene signature (poor survival gene signature) using microarray analysis [71]. Some of the relevant signatures include those discovered by Boyault et al., where 193 HCC patients were categorized into subgroups according to 6 different gene signature classes. A "G3" signature was suggestive of worse prognosis compared with other classes. The G3 signature included the following genes: FAL, p53, LOH 17p, LOH 16p, LOH 4q, LOH

5q, LOH 21q, and LOH 22q [72]. Villanueva et al. revisited 22 previously studied gene signatures that were reported to be significantly prognostic. Using tumors from 287 HCC patients, the investigators measured and compared the prognostic power of each signature [73]. Following multivariate analysis, the G3 signature, and another "poor-survival" signature were the only ones to predict a higher risk of HCC recurrence in resected subject (HR = 1.75 [P = 0.003] and HR = 1.74 [P = 0.004], respectively) [73]. On the other hand, Nault et al published a "5-gene score" after reviewing 314 tumor samples using 18 genetic signatures selected based on literature review. First, a microarray method was applied to construct the genomic data from 44 patients, which defined 103 genes commonly mutated in HCC. Then a statistical model was applied to define the mutated genes that could predict recurrence. The selected apparently predictive genes then underwent validation in a different cohort of 314 subjects with HCC who were treated by tumor resectio n [70]. The validated genes, making up the 5-gene score, were 1-TATA box-binding protein associated factor 9 **(TAF9)**, which functions as mediator of transcription activation and a cell cycle regulator [74]; 2-RAS related nuclear protein **(RAN)**, which plays a role during mitosis and spindle and microtubule assembly [75]; 3-Receptor activity modifying protein **(RAMP3)**, which functions as a part of the hormone receptor system for calcitonin or adrenomedullin [76]; 4-Notch1 or HN1, which is a member of the Notch signaling pathway membrane protein family, and plays a role in neovascularization [77]; and 5-Keratin 19 **(KRT19)**, which is a member of the keratin protein family that maintains stem cell integrity [78]. This 5-gene score was associated with worse disease-specific survival (HR = 2.93, 95% CI: 1.8 – 4.66; p-value < 0.0001) [70]. However, the differences between the different gene signatures could be related to differences in predisposing factors, the method of lab testing, and the population studied.

miRNA signatures in HCC

miRNAs mediate post-transcriptional silencing of target genes, regulate the expression levels of certain genes in tumor cells, and down-regulate tumor suppressor genes [79,80]. miRNA measurement can be used as predictive tool for HCC. As an example, abnormalities involving β-catenin, part of the WNT pathway, can be assessed through miRNAs (miR-21, -122, -124, -135, -192, -200, -315, 331-3p). Another example is C-MET, which is regulated by miR-1, miR-23b, miR-34a, and miR-199-3p. The C-MET pathway can also be upregulated by miR-221 and miR-222, through c-Jun transcription factor, leading to down-regulation of PTEN and TIMP3, and resistance to TRAIL induced cell death [81].

Utilizing microarray technology, Sato et al reported the association of miRNA testing in HCC with survival and recurrence. HCC and non-tumor tissue specimens from 73 patients who underwent resection were tested. Higher expression of miRNA in tissue surrounding the tumor was associated with higher HCC recurrence, suggesting a disturbance in oncogenic miRNA regulation in this surrounding tissue (Table 1) [82].

Table 1: Contribution analysis of individual miRNAs in the overall best prediction model of HCC recurrence.

	microRNAs positively associated with recurrence			microRNAs negatively associated with recurrence		
Rank	miR name	Tissue type	Coefficient	miR name	Tissue type	Coefficient
1	miR-96	N	0.1349	miR-96	T/N ratio	−0.0865
2	miR-139-5p	T/N ratio	0.0750	miR-374b	T/N ratio	−0.0785
3	miR-139-5p	T	0.0727	miR-182	T/N ratio	−0.0604
4	miR-126*	T	0.0676	miR-378	N	−0.0561
5	miR-142-3p	T	0.0673	miR-193b	T/N ratio	−0.0543
6	miR-142-3p	T/N ratio	0.0659	miR-193b	T	−0.0539
7	miR-362-3p	T	0.0596	miR-214	T/N ratio	−0.0535
8	miR-374b	N	0.0563	miR-125b	T	−0.0522
9	miR-10b	T	0.0548	miR-125b	T/N ratio	−0.0521
10	miR-200a	N	0.0544	miR-99b	T/N ratio	−0.0517
11	miR-224	N	0.0530	miR-1202	N	−0.0515
12	miR-483-3p	T	0.0529	miR-18b	T/N ratio	−0.0513
13	miR-200a	T	0.0527	miR-365	T/N ratio	−0.0511
14	miR-1202	T/N ratio	0.0507	miR-100	T/N ratio	−0.0506
15	miR-96	T	0.0484	miR-365	T	−0.0504
16	miR-665	T/N ratio	0.0474	miR-210	T	−0.0502
17	miR-1274a	T/N ratio	0.0472	miR-100	T	−0.0500
18	miR-10b	T/N ratio	0.0471	miR-214	T	−0.0491
19	miR-665	T	0.0469	miR-378	T	−0.0489
20	miR-1228	T/N ratio	0.0446	miR-182	T	−0.0476
T= tumor tissue, N= non-tumor tissue						

Role of Circulating Cancer Stem Cells in Predicting HCC Recurrence

Cancer stem cells **(CSCs)** have special features, including self-renewal, generation of progeny, and resistance to chemotherapy and radiotherapy [83]. CSCs in HCC are thought to express epithelial cell adhesion molecule **(EpCAM)** [84]. Hence, EpCAM cells can be used as a surrogate for metastasis and a prognostic marker for HCC [84]. Schulze et al demonstrated the relationship between the presence and the quantity of EpCAM cells in the serum and HCC prognosis. The mOS of HCC patients with EpCAM-positive results (≥ 1 CTC in 7.5 mL) was 460 days (95% CI: 70–850 days), which was significantly less than the 746 days (95% CI: 572–919 days) seen for patients without EpCAM cells in the serum (P = 0.017) [85].

Role of Immune Microenvironment in Cancer Recurrence

Yang et al described the HCC microenvironment as a dynamic interaction between cellular and non-cellular components. The cellular components mainly include hepatic stellate cells, fibroblasts, immune cells, and endothelial cells. The non-cellular components (produced by the

cellular components) include the extracellular matrix **(ECM)** proteins, protolytic enzymes, growth factors, and inflammatory cytokines. This cellular and non-cellular component-interaction plays a range of roles, from activating signalling pathways, to promoting invasion and metastasis [86]. In the last 2 decades, various components of the HCC microenvironment have been studied for their possible use in disease prognosis and predicting treatments effects.

Tumor-Infiltrating Cells

The presence of tumor infiltrating lymphocytes **(TILs)** is an indication of host reaction against the tumor [87]. CD4+, CD25+, and FOXP3+ regulatory T cells **(Tregs)** function as immunosuppressants. Kobayashi et al demonstrated the relationship between Treg infiltration and survival in 325 HCC tumors (various stages). Higher infiltration of Tregs was associated with lower survival (HR = 1.64, 95% CI = 1.023-2.628; P = 0.04) [88]. This was regardless of the presence of CD8+ cytotoxic cells [88]. Mathai et al demonstrated worsening OS in patients with an increased Foxp3:CD8 ratio (HR=1.153, 95% CI = 1.019-1.304; P = 0.0235) [89]. Tumor-associated macrophages **(TAMs)** are macrophages that infiltrate tumor tissues; they serve different functions within the tumor depending in their class. Shu et al demonstrated better OS with high CD11c+ and low CD206+ TAMs, based on the analysis of 80 HCC tissue samples [90]. Similar work suggested an association between mesenchymal stem cells **(MSC)**, neutrophil tumor infiltration, and HCC outcomes [91,92].

CXC ligand **(CXCL)** molecules **(Chemokines)** function as immune cell regulators. Li et al demonstrated a correlation between the outcome of 227 patients with resected HCC and expression of a novel chemokine, CXCL17. Increased peritumoral CXCL17 expression was associated with worse OS (HR=2.06, 95% CI = 1.29-3.29; P = 0.002) [93]. Similarly, high expression of another chemokine, CXCR2, was associated with OS (HR = 1.737, 95% CI = 1.69-2.58; P = 0.006) [91]. Programmed cell death 1 (PD-1), is a member of the CD28 immunoinhibitory receptor family. Gao et al, demonstrated worse survival with high PD-1 expression (HR = 1.61, 95% CI = 1.04-2.50; P = 0.032) [94].

Chen et al (45), demonstrated a relationship between the presence of certain cytokines in the serum and DFS. Among thirty-nine cytokines that were analyzed in patient serum, six of them had a significant relationship with DFS, namely fibroblast growth factor 2 **(FGF-2)**, growth-regulated oncogene **(GRO)**, interleukin 8 (IL-8), interferon gamma-induced protein 10 (IP-10), VEGF, and interferon alpha-2 **(IFN-α2)** [95].

SUMMARY

Several clinicopathological risk factors associated with HCC recurrence after liver resection have been studied, which can be broadly categorized into tumor, host, and surgical causes. Vascular invasion is one of the strongest predictors of recurrent disease, which serves as an independent predictor for numerous other clinical factors. Molecular and cellular predictors for cancer recurrence including, but not limited to, deregulated pathways and genes, miRNAs,

circulating cancer stem cells, tumor-infiltrating cells, are emerging and been evaluated in clinical studies. Understanding the pathogenesis of HCC at the cellular and molecular level may lead to strategies to prevent cancer recurrence.

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