Review

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Exploration of Practical Biomarkers for Systemic Drug Therapy for Hepatocellular Carcinoma

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Simple Summary: A number of agents, including immune checkpoint inhibitors, have become available for the treatment of hepatocellular carcinoma, but the objective response rate of these drugs is currently only 30% to 40%. Therefore, the identification of new predictive biomarkers and an increased knowledge of the mechanisms of response or resistance to systemic chemotherapies are required.

Abstract: A number of agents, including immune checkpoint inhibitors, have become available for the treatment of hepatocellular carcinoma (HCC). However, the objective response rate of these drugs is currently only 30% to 40%, with a high incidence of side effects. There are also no practical biomarkers to predict their therapeutic effects. Most of the systemic therapies for HCC are performed in general hospitals without research facilities. Such hospitals can perform imaging tests, like CT and MRI, as well as pathological diagnosis using tumor tissue sampling and immunohistochemical staining. However, analyzing tumor genomic or transcriptomic profiles is difficult because of limitations in facilities, personnel, and cost. Therefore, in this review, we provide an overview of the wide range of research that has been conducted on HCC biomarkers from blood, tissue, or imaging information that can be used practically in general hospitals for predicting the therapeutic effect of systemic therapies before treatment begins. For general hospitals that treat HCC patients, we recommend conducting treatment after assessing the state within the tumor tissue as much as possible by collecting blood and tissue samples and performing pre- treatment MRI image evaluations.

Keywords: hepatocellular carcinoma; tyrosine kinase inhibitors; immune checkpoint inhibitors; biomarkers

1. Introduction

Liver cancer is a major cause of death worldwide, and the number of people diagnosed with liver cancer is expected to increase [1]. As the principal histologic type of liver cancer, hepatocellular carcinoma (HCC) is responsible for the great majority of liver cancer diagnoses and deaths, accounting for approximately 75% of the total [2]. Treatment options for localized HCC, such as surgical resection, ablation, liver transplantation, and transarterial chemoembolization (TACE) , were established in the 20th century, but an effective drug therapy for advanced HCC did not emerge until 2007 [3]. Although many clinical trials of potential drug therapies for unresectable HCC were conducted before the introduction of tyrosine kinase inhibitors (TKIs), no chemotherapeutic drugs demonstrated any significant survival benefit, as shown in the meta-analysis by Mathurin et al. [4]. Subsequently, an advanced understanding of the mechanisms of tumor cell proliferation and angiogenesis supported the development of the TKI sorafenib [5]. In the clinical trial "SHARP trial" for unresectable HCC, sorafenib showed a clear survival benefit over placebo and became the standard treatment for unresectable HCC in 2007 [6]. Subsequently, the results of the "RESORSE trial", a clinical trial limited to unresectable HCC patients who have tolerated sorafenib led to the approval of regorafenib as a second-line therapy after sorafenib treatment in 2017 [7]. In 2018, lenvatinib demonstrated "non-inferiority" to sorafenib in the "REFLECT trial", leading to a choice of either sorafenib or lenvatinib as the first-line therapy [8]. In 2019, based on the results of the "REACH-2 trial" for cases with alpha-fetoprotein (AFP) levels of 400 ng/mL or more after sorafenib treatment,

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ramucirumab (an anti-vascular endothelial growth factor (VEGF) receptor antibody) became available as a second-line therapy [9]

In the following years, immune checkpoint inhibitors (ICIs) emerged. In 2020, a combination therapy of atezolizumab and bevacizumab, which surpassed sorafenib in clinical trials, was introduced [10]. As a result, both sorafenib and lenvatinib were relegated to second-line or later therapies. Furthermore, a combination therapy of two ICIs, durvalumab and tremelimumab (STRIDE regimen), outperformed sorafenib in treatment results of the "HIMALAYA trial". Durvalumab monotherapy also showed non-inferiority to sorafenib [11], allowing these therapeutics to be added as new treatment options. Additionally, a TKI, cabozantinib, was developed as a second-line or later therapy [12].

Through these trials, a number of agents, including ICIs, have become available for treating HCC. However, the objective response rate (ORR) of these drugs is currently only 30% to 40%, with a high incidence of side effects [6–12]. Other than ramucirumab, which has the condition of AFP 400 ng/mL or more, there are no practical biomarkers to predict the therapeutic effects of these treatment methods.

Most of the systemic therapies for HCC are performed in general hospitals without research facilities. In such hospitals, it is possible to perform imaging tests, such as CT and MRI, and pathological diagnosis, including tumor tissue sampling and immunohistochemistry (IHC) staining. However, analyzing the tumor genomic or transcriptomic profiles is often difficult because of limitations in facilities, personnel, and cost, as well as increased medical costs. Therefore, in this review, we provide an overview of the wide range of research that has been conducted on HCC biomarkers from blood, tissue, or imaging information that can be used practically in general hospitals for predicting the therapeutic effect of systemic therapies before treatment begins. We also examine and describe future prospects in this field.

2. Exploring Biomarkers for Predicting the Therapeutic Effects of TKIs (Table 1)

2.1. Sorafenib Biomarkers

The first TKI, sorafenib, has remained the first-choice drug for a decade, leading to numerous early studies being conducted on biomarkers for predicting its therapeutic effect.

One of the objectives of the Phase III SHARP (Sorafenib HCC Assessment Randomized Protocol) trial was to investigate plasma biomarkers for predicting prognosis and therapeutic effect, with 10 plasma biomarkers measured at baseline and after 12 weeks of treatment [13]. As a result, baseline angiopoietin 2 (Ang-2) and VEGF concentrations were found to be independent predictors of survival in the entire advanced HCC patient population. However, these were not unique to the sorafenib cohort and were similar in the placebo cohort [13].

In a real-world study in Japan, Miyahara et al. measured the serum levels of eight pro-angiogenic cytokines (Ang-2, follistatin (FST), granulocyte colony-stimulating factor (G-CSF), hepatocyte growth factor (HGF), leptin, platelet-derived growth factor-BB (PDGF-BB), platelet endothelial cell adhesion molecule-1 (PECAM-1), and VEGF) in 120 consecutive HCC patients treated with sorafenib. They reported that high expression of Ang-2 or high expression of three or more pro-angiogenic cytokines was associated with poor progression-free survival (PFS) and overall survival (OS) in patients treated with sorafenib [14]. In addition, the presence of macrovascular invasion (MVI) was also shown to be associated with poor OS in clinical parameters [14].

Table 1. Factors influencing patient prognosis or efficacy for treating hepatocellular carcinoma with tyrosine kinase inhibitors.

Agent	Study design	Number of cases	Prognostic and predictive factors	Out-come	Statistical analysis	HR [95% CI]	*P*-value	Authors [reference no.]
Sora-fenib	Retrospective, single-arm	120	[High serum Ang-2]	PFS OS	Univariate Multivariate	1.84 [1.21–2.81] 1.83 [1.12–2.98]	0.004 0.014	Miyahara K et al. [14]

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			[High angiogenic group*] *: patients with > three serum cytokines (Ang-2, FST, G-CSF, HGF, Leptin, PDGF-BB, PECAM-1, or VEGF)	PFS OS	Univariate Multivariate	1.98 [1.30–3.06] 1.76 [1.07–2.94]	0.001 0.023	
				OS	Multivariate	2.27 [1.36–3.72]	0.001	
			[MVI (present)]					
Sora-fenib	Retrospective pooled analysis of two phase 3 trials (vs. placebo)	Sorafenib 448 Placebo 379	[Without EHS] [With HCV] [Low NLR]	OS OS OS	Multivariate Multivariate Multivariate	0.55 [0.42–0.72] 0.47 [0.32–0.69] 0.59 [0.46–0.77]	0.015 0.035 0.0497	Bruix J et al. [15]
Sora-fenib	Subgroup meta-analyses, single-arm	170	[Low NLR]	OS	Univariate	1.49 [1.17–1.91]	0.001	Qi X et al. [18]
Sora-fenib	Observational registry, single-arm	3,371	[Child-Pugh A] [Bilirubin] [Albumin]	OS OS OS	Kaplan-Meier Univariate Univariate	- 1.71 [1.57–1.86] 1.76 [1.63–1.89]	N/A N/A N/A	Marrero JA et al. [19]
Sorafe-nib	Retrospective, single-arm, HCV patients only	103	[HCV eradication] [ALBI score]	OS OS	Multivariate Multivariate	0.46 [0.26–0.78] 2.29 [1.20–4.37]	0.004 0.012	Kuwano A et al. [20]
Sora-fenib	Population-based retrospective cohort, HCV patients only, single-arm	1,684	[DAA user]	OS	Univariate PSM univariate	- - -	< 0.0001 < 0.0001	Tsai H-Y et al. [21]
Sora-fenib	Retrospective, single-arm	55	[FGF3/FGF4amplification] (frozen tumor tissue)	CR/PR	Fisher's exact	-	0.006	Arao T et al. [26]
			[multiple lung metastases]	CR/PR	Fisher's exact	-	0.006	
Sora-fenib	Retrospective, single-arm	20	[High miR-224 expression] (FFPE tumor tissue)	PFS OS	Univariate Univariate	0.28 [0.09–0.92] 0.24 [0.07–0.79]	0.029 0.012	Gyöngyösi B et al. [28]
Sora-fenib	Retrospective, single-arm	Training coh. 26 Valid. coh. 58	[High miR-425-3p expression] (FFPE tumor tissue)]	TTP PFS	Multivariate Multivariate	0.4 [0.1–0.7] 0.3 [0.1–0.7]	0.002 0.0012	Vaira V et al. [31]
Sora-fenib	Retrospective validation of the pharmacogenomics panel, single-arm	54	[High serum DKK-1]	PFS OS	Univariate Univariate	- -	0.0396 0.0171	Qiu Z et al. [33]

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Regora-fenib	Retrospective pooled analysis of the phase 3 trial (vs. placebo)	Protein cohort Regora 332 Placebo 167	[Plasma ANG-1] (1 ng/mL increase)	OS	Multivariate	1.12 [1.05–1.19]	0.019	Teufel M et al. [35]
			[Low plasma Cystatin-B] (2-fold increase)	TTP	Multivariate	1.10 [1.04–1.17]	0.017	
			[Low plasma LAP TGF-β1] (2-fold increase)	OS	Multivariate	1.46 [1.15–1.85]	0.04	
				TTP	Multivariate	1.42 [1.14–1.77]	0.018	
				OS	Multivariate	1.36 [1.12–1.65]	0.04	
			[Low plasma LOX-1] (1 ng/mL increase)	TTP	Multivariate	1.41 [1.18–1.68]	0.004	
				OS	Multivariate	1.35 [1.16–1.57]	0.009	
			[Low plasma MIP-1α] (1 pg/mL increase)	TTP	Multivariate	1.78 [1.33–2.39]	0.003	
				OS	Multivariate	1.02 [1.01–1.04]	0.04	
				TTP	Multivariate	1.02 [1.00–1.03]	0.043	
		miRNA cohort Regora 234 Placebo 109	[miR-15b]	OS	Multivariate	0.37 [0.20–0.70]	0.002	
			[miR-107]	OS	Multivariate	0.54 [0.37–0.81]	0.003	
			[miR-320b]	OS	Multivariate	0.57 [0.41–0.81]	0.001	
			[miR-122]	OS	Multivariate	1.35 [1.14–1.60]	0.0004	
			[miR-374b]	OS	Multivariate	1.36 [1.11–1.65]	0.002	
			[miR-200a]	OS	Multivariate	1.39 [1.15–1.68]	0.001	
			[miR-30a]	OS	Multivariate	1.47 [1.14–1.88]	0.003	
			[miR-125b]	OS	Multivariate	1.54 [1.19–1.99]	0.001	
			[miR-645]* (*dichotomized analysis, not vs. placebo)	OS	Multivariate	3.16 [1.52–6.55]	0.002	
Lenva-tinib	Subgroup analysis of the open-label phase 3 trial (vs. sorafenib)	Lenvatinib 478 (HBV 251, Alcohol 36) Sorafenib 476 (HBV 228, Alcohol 21)	[HBV]	PFS	Univariate	0.62 [0.50–0.75]	N/A	Kudo M et al. [8]
			[Alcohol]	PFS	Univariate	0.27 [0.11–0.66]	N/A	
Lenva-tinib	Retrospective, single-arm	237	[NLR ≥ 4]	OS	Multivariate	1.87 [1.10–3.12]	0.021	Tada T et al. [53]
				PFS	Multivariate	1.90 [1.27–2.84]	0.002	
			[AFP ≥ 400 ng/mL] [mALBI grade 2b or 3] [BCLC stage ≥ C]	DCR	Chi-square test?		0.007	
				OS	Multivariate	1.97 [1.19–3.27]	0.009	
				OS	Multivariate	2.12 [1.27–3.56]	0.004	
				PFS	Multivariate	1.52 [1.03–2.24]	0.036	
Lenva-tinib	Retrospective, single-arm	1,325	[HBV]	OS	Multivariate	1.56 [1.13–2.17] *	0.0071*	Casadei-Gardini A et al. [54] *: data are from the model 1 of 3 multivariate analyses.
			[NAFLD/NASH]	OS	Multivariate	0.58 [0.33–0.98] *	0.0044*	
				PFS	Multivariate		0.0090	
			[BCLC stage C]	OS	Multivariate	0.87 [0.75–0.93]	0.0027*	
				PFS	Multivariate	1.64 [1.19–2.27]	0.0002	
			[NLR > 3]	OS	Multivariate	*	< 0.0001*	
				PFS	Multivariate	1.33 [1.14–1.55]	0.0482	
			[AST > 38]	OS	Multivariate	1.95 [1.46–2.60]	0.0167*	
				PFS	Multivariate	*	0.0365	
						1.16 [1.01–1.36]		

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						1.52 [1.08–2.13] * 1.21 [1.01–1.45]		
Lenva-tinib	Retrospective validation of the experimentally identified biomarker (vs. sorafenib)	Lenvatinib 65 (ST6GAL1 high 22, low 43) Sorafenib 31 (ST6GAL1 high 12, low 19)	[Serum ST6GAL1 high]	OS	Univariate		< 0.05	Myojin Y et al. [55]

HR, hazard ratio; CI, confidence interval; PFS, progression-free survival; OS, overall survival; CR, complete response; PR, partial response; TTP, Time to Progression; DCR, disease control rate; HBV, hepatitis B virus; HCV, hepatitis C virus; MVI, macrovascular invasion; EHS, extrahepatic spread; NLR, neutrophil to lymphocyte ratio. Note: In the 'Statistical analysis' section, 'univariate' typically refers to the Kaplan-Meier method and log-rank test, while the inclusion of HR indicates the use of Cox regression. Additionally, 'multivariate' typically refers to the utilization of the multivariate Cox regression model.

In an analysis of pooled data from the SHARP and Asia Pacific (AP) Phase III trials, a significantly greater OS benefit compared with placebo was observed in patients without extrahepatic spread (EHS; hazard ratio (HR), 0.55 vs. 0.84), with hepatitis C virus (HCV) (HR, 0.47 vs. 0.81), and a low neutrophil to lymphocyte ratio (NLR) (HR, 0.59 vs. 0.84) [15]. In this analysis, the NLR was divided into more than 3.0 and less than 3.0, which was the median in the sorafenib administration group. Although this was a retrospective study, it described certain factors that had significant differences compared with the placebo group. However, these findings were strictly predictions of the survival period and not of drug effectiveness.

The NLR reflects the inflammatory response to cancer, and its elevation is recognized as an indicator of poor prognosis [16,17]. Qi et al. conducted a systematic review and meta-analysis of 20,475 HCC patients from 90 articles to explore the prognostic role of NLR in HCC, finding that a lower baseline NLR was significantly associated with the survival period (HR, 1.80, 95% confidence interval (CI): 1.59–2.04, $P < 0.00001$) [18]. This study also conducted a subgroup meta-analysis for cases treated with sorafenib, and although it was not compared with a placebo group, low NLR was associated with better survival in 170 cases between high and low NLR groups [18].

The GIDEON trial, a large prospective observational registration study focused on assessing the safety of sorafenib treatment in the real world, showed better OS in Child-Pugh A patients compared with Child-Pugh B patients. Moreover, a univariate Cox regression analysis of each factor of the Child-Pugh score showed that albumin and bilirubin, which form the ALBI score, strongly influenced OS [19]. The authors did not utilize anything other than descriptive statistics that considered the impact of selection bias. Additionally, this study lacked a control group and was not randomized. However, liver functional reserve can clearly affect the patient survival period after treatment with sorafenib for HCC. For example, several studies have shown that in advanced HCC cases related to HCV, OS is extended as liver functional reserve improves following HCV eradication with interferon (IFN) or direct-acting antivirals (DAA) [20,21].

For pathological biomarkers in tumor tissues, the Phase II trial results indicated that phosphorylated ERK could potentially be useful as a biomarker for predicting the prognosis of patients treated with sorafenib. This protein is located downstream of the Raf kinase in the MAPK cascade, which is a major target of sorafenib [22]. While there have been studies in the clinical setting that have shown favorable efficacy [23], several have also shown unfavorable efficacy [24,25]. Therefore, no consensus has been reached regarding phosphorylated ERK.

Arao et al. reported that in 13 cases that showed significant tumor shrinkage with sorafenib treatment, fibroblast growth factor (FGF)3/FGF4 amplification was observed in the tumor genome. Additionally, multiple lung metastases in poorly differentiated histological types were seen as

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clinical pathological features. Although the sample size was relatively small, FGF3/FGF4-amplified tumors were frequently observed in responders to sorafenib [26]. Although this study examined biomarkers for sorafenib treatment efficacy, no further research with additional cases to support these findings was conducted.

Tumor tissues can also be used to investigate microRNA (miRNA) expression. MiRNAs are small endogenous non-coding RNAs that can inhibit translation or support cleavage of mRNAs to negatively regulate gene expression. These molecules are highly stable and can be reliably detected in stored clinical samples and cell cytology specimens, making them ideal biomarker candidates [27–29]. Various miRNAs are also mechanistically involved in the development, proliferation, and progression of HCC and can be detected in serum and plasma samples, suggesting they could potentially be used as diagnostic markers [30].

Gyöngyösi et al. investigated the expression levels of 14 miRNAs in 20 HCC cases where tumor tissue samples were collected by fine needle aspiration before sorafenib administration, with the data demonstrating that high expression of miR-224 was associated with increased PFS and OS rates [28].

Vaira et al. conducted a comprehensive profiling of approximately 700 miRNAs in a series of 26 HCC patients treated with sorafenib (training set) using tumor tissues collected prior to treatment, then verified the results in an independent series of 58 patients (validation set) [31]. As a result, six miRNAs were found to be significantly associated with clinical variables in the training set. Of these, only miR-425-3p was significant in the validation set, with high miR-425-3p levels being associated with longer time to progression (TTP) and PFS [31]. However, no follow-up studies have been conducted. In general, miRNA-related cancer research is primarily focused on the development of therapies that target specific miRNAs or use these molecules as a tool for early cancer detection [32].

Other studies have constructed high-throughput assay systems in completely different ways. Qiu et al. created a Liver Cancer Model Repository (LIMORE) panel of 81 cell lines by creating 50 patient-derived liver cancer cell lines, in addition to 31 existing cell lines, to model HCC heterogeneity. The authors examined the sensitivity of the cells to a total of 90 drugs. By using this panel, which has verified gene mutations and gene expression characteristics, it is possible to identify gene-drug interactions of therapeutic methods and biomarker candidates. When predicting the effect of sorafenib treatment, a molecule called Dickkopf-1 (DKK-1) was identified as a potential useful biomarker [33]. Interestingly, DKK-1 is a secreted protein that antagonizes Wnt signal transduction, which is known to affect ICI efficacy [34]. Because DKK-1 is a serum protein, it may be relatively easy to verify its potential as a biomarker in existing cohorts with preserved serum samples.

2.2. Regorafenib Biomarker Studies

Although insufficient results were obtained from the biomarker studies in the Phase III SHARP trial for sorafenib, a far more comprehensive exploratory biomarker analysis was conducted for patients in the RESORCE trial at the DNA, RNA, and protein levels [35]. Of the 266 proteins studied in baseline plasma samples, decreases in five, Ang-1, cystatin B, the latency-associated peptide of transforming growth factor beta 1 (LAP TGF-β1), oxidized low-density lipoprotein receptor 1 (LOX-1), and C-C motif chemokine ligand 3 (MIP-1α), were found to be associated with extended TTP and OS. Moreover, nine plasma miRNAs, miR-30a, miR-122, miR-125b, miR-200a, miR-374b, miR-15b, miR-107, miR-320, and miR-645, were related to OS, although none were associated with TTP. Furthermore, there was no apparent correlation between the AFP or c-MET protein expression levels and the OS or TTP benefits of regorafenib treatment, causing them to be excluded as potential predictive biomarkers [35]. Currently, with treatments including ICIs becoming the standard of care as first-line treatments, the number of cases where regorafenib is used after sorafenib treatment is likely not substantial. This could make planning prospective validation studies challenging. However, as these potential biomarkers are plasma proteins and miRNAs, it is hoped that they can be validated in existing cohorts using blood samples that have been preserved.

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2.3. Signaling Pathways as Biomarkers for TKIs: Insights from Trials with mTOR and MET Inhibitors

In several cancer types other than HCC, there are driver gene mutations in signaling pathways that strongly promote tumor growth. This has led to established biomarker-driven treatment concepts for drug selection and predicting treatment outcomes [36–38]. In HCC, signaling pathways such as RAS, mammalian target of rapamycin (mTOR), MET, and FGF-19 have been considered as potential therapeutic targets. Several clinical trials were conducted using inhibitors targeting these pathways, but many unfortunately ended with disappointing results [39]. For example, mTOR signaling is activated in about half of all HCC cases and is associated with worse outcomes [40]. Despite this strong theoretical basis for using the mTOR inhibitor everolimus for treating HCC, the final results from a phase III trial did not suggest any trend of prolonged OS (everolimus vs. placebo, 7.6 vs. 7.3 months) [41].

The potential cause of failure in these clinical trials may be the inclusion of all patients with unresectable HCC. It has been considered desirable to incorporate molecular selection factors into prospective research, as in clinical trials for other cancers [42]. One of the drugs for which there was hope for biomarker-driven treatment in HCC was tivantinib. Tivantinib, a MET inhibitor, was investigated in a phase II trial against placebo as a second-line treatment. Although no significant effect was observed in all cases, it improved survival rates in patients with high tumor MET expression levels [43]. From these results, phase III trials (METIV-HCC, JET-HCC) were conducted comparing tivantinib and placebo only in patients with high MET expression [44,45]. However, in both trials, no statistically significant treatment effect was observed for tivantinib compared with placebo.

Cabozantinib, which targets several TKs including MET, VEGF, and AXL, was successful in a phase III trial (CELESTIAL) [12]. In this trial, baseline plasma levels of MET, AXL, VEGFR2, HGF, GAS6, VEGF-A, PlGF, IL-8, EPO, ANG2, IGF-1, VEGF-C, and c-KIT were evaluated as biomarkers, but none of them could predict the treatment effect of cabozantinib on OS or PFS [46].

HCC has diverse causes, including viral infection, toxin exposure, and metabolic disorders. From the results of large-scale genomic analyses, it has become clear that gene mutations in HCC are centered on non-drug targetable mutations, such as TERT, CTNNB1, TP53, that are diverse [47,48]. Furthermore, HCC is heterogeneous even within an individual patient, and sequencing analysis of a single lesion cannot fully characterize the genomic features of HCC in certain cases [49]. The failure to use the expression patterns of specific therapeutic target molecules or activated signaling as biomarkers in TKI clinical trials may indirectly suggest that the heterogeneity and diversity of HCC results in a lack of full dependence on the several signaling pathways that have been considered [50].

2.4. New Approaches for Biomarker Discovery in Lenvatinib Treatment

Lenvatinib is a multi-kinase inhibitor that inhibits vascular endothelial growth factor receptors (VEGFR) 1–3, fibroblast growth factor receptors (FGFR) 1–4, platelet-derived growth factor receptor (PDGFR) α, and oncogenes RET and KIT [51]. Preclinical studies have shown that lenvatinib has strong anti-angiogenic activity, primarily through inhibition of the VEGF and FGF signaling pathways [52].

In a subgroup analysis of the REFLECT trial, patients with HBV infection or alcohol as underlying factors showed better PFS rates with lenvatinib treatment than with sorafenib [8]. However, it seems that no biomarker exploration beyond subgroup analysis was planned in the REFLECT trial.

Tada et al. focused on the associations between outcomes in HCC patients treated with lenvatinib and the NLR. In a multivariate analysis of a cohort of 237 individuals, an NLR ≥ 4 was independently associated with OS and PFS. There was also a significant difference in the disease control rate between patients with low NLR (< 4) and high NLR (≥ 4) (85.5% vs. 67.3%, P = 0.007). A spline curve analysis showed that an NLR of approximately 3.0 to 4.5 was an appropriate cutoff value related to OS (53). In addition, in the retrospective RELEVANT study from 23 other facilities, data were collected for 1,325 patients treated with lenvatinib. In the multivariate analysis of OS, HBsAg positivity, NLR > 3, and AST > 38 were independently associated with poor prognosis in all three

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models. Furthermore, nonalcoholic fatty liver disease (NAFLD)/ nonalcoholic steatohepatitis (NASH) -related etiology was independently associated with a good prognosis. The multivariate analysis showed that NAFLD/NASH, Barcelona Clinic Liver Cance (BCLC) stage , NLR, and AST were independent prognostic factors for PFS in cases treated with lenvatinib [54]. However, these studies did not make comparisons with placebo or other drug treatments. Essentially, a control group or placebo group is necessary to identify predictors of the therapeutic effect of a certain drug, so it is necessary to consider the limitations of such single-arm observational studies when evaluating predictive markers.

Qiu et al., who identified DKK-1 as a potential predictor of sorafenib effectiveness, confirmed with their panel of 81 HCC cell lines, LIMORE, that FGFR inhibitors like lenvatinib have a favorable effect on HCC strains with amplification of FGFR and FGF. They suggested that the amplification of FGF19 and FGFR could potentially serve as biomarkers for lenvatinib effectiveness [33].

Myojin et al. developed a new HCC mouse model that reproduces the diversity of tumor driver genes by introducing a pooled cancer gene cDNA library using transposon-based intrahepatic delivery. This could be used to simultaneously evaluate the individual effects of various genetic drivers on TKI sensitivity in HCC in vivo. This model revealed that tumors expressing FGF19 are sensitive to lenvatinib in vivo. They comprehensively evaluated tumor secretory proteins to discover biomarkers for FGF19-driven HCC, identifying a correlation between FGF19 and the secretory protein ST6 β-galactoside α-2,6-sialyltransferase 1 (ST6GAL1) in HCC cells. This provided clinical evidence that ST6GAL1 may be a useful serum biomarker for selecting HCC patients who may benefit more from lenvatinib than sorafenib treatment [55]. This study strongly focused on the exploration of biomarkers for the therapeutic effect of lenvatinib in cancer cells, but validation in a prospective cohort is necessary for clinical application. Because ST6GAL1 protein levels can be measured in serum samples, it would be a very useful biomarker if validated.

Lenvatinib reportedly has higher selectivity for FGFR compared with other kinase inhibitors [51,56]. Therefore, biomarkers related to FGF-FGFR signaling may be more promising than those related to other signaling pathways that have been previously studied.

3. AFP as a Predictive Biomarker for Ramucirumab Treatment

VEGF and VEGFR2 signaling plays a crucial role in angiogenesis and tumor growth [57]. Multi-kinase inhibitors, such as sorafenib and lenvatinib that have been shown to be effective against HCC, always target VEGFR2. Ramucirumab is a human IgG1 monoclonal antibody that inhibits ligand activation of VEGFR2 [58]. In a Phase II trial of ramucirumab as a first-line therapy for HCC, ramucirumab demonstrated an overall response rate (ORR) and OS that surpassed the sorafenib administration group in the SHARP trial [59]. In this trial, an exploratory study of biomarkers measured circulating VEGF, soluble VEGFR1 (sVEGFR1), sVEGFR2 , and several cytokines and growth factors in serum samples after ramucirumab administration. Among them, a potential correlation was suggested between reduced serum sVEGFR1 levels until day 8 post-administration and prolonged PFS and OS [58]. However, in the 'REACH trial', a Phase III trial of ramucirumab vs. placebo as a second-line therapy after sorafenib treatment, a significant improvement in OS was not achieved in the ramucirumab group compared with the placebo group [60].

Apart from the initially explored biomarkers, a subgroup analysis of the REACH trial revealed that OS in the ramucirumab group was significantly better when limited to cases with AFP ≥ 400 ng/mL [60]. Therefore, the 'REACH-2 trial' was planned, which was a Phase III trial of ramucirumab vs. placebo as a second-line therapy restricted to cases with AFP ≥ 400 ng/mL after sorafenib treatment [9]. As expected, the REACH-2 trial results indicated that OS was significantly extended in the ramucirumab treatment group compared with the placebo group. This trial became the first successful Phase III trial for advanced HCC treatment that selected target cases using a biomarker [9].

Because AFP was shown to be a predictive biomarker for the therapeutic effect of ramucirumab, to investigate the molecular profile differences of tumors using AFP levels, an analysis of 520 HCC

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cases with known baseline AFP values was conducted. The data suggested that tumors in cases with AFP > 400 ng/mL showed significant activation of VEGF signaling [61].

4. Exploration of Biomarkers for Predicting the Therapeutic Efficacy of Single-agent ICIs and Combined Immunotherapy (Table 2)

The pharmacotherapy of HCC has shifted from being dominated by TKIs to ICIs and combined immunotherapies. Correspondingly, research into biomarkers for predicting therapeutic effectiveness has transitioned from focusing on those related to tumor growth signals to those focusing on the tumor microenvironment and tumor immune environment [62].

Studies on biomarkers for predicting the therapeutic effects of ICIs for HCC began with the validation of biomarkers discovered in other cancer types, such as melanoma, non-small cell lung cancer, and colorectal cancer. However, despite the demonstrated response rate of about 20% for HCC cases to single-agent therapies such as nivolumab and pembrolizumab, these treatments could not become standard first-line or second-line therapies [63–67]. The established combination therapy of atezolizumab and bevacizumab, which are anti-PD-L1 and anti-VEGF-A antibodies, respectively, became the standard treatment. This resulted in research on biomarkers for predicting the therapeutic effects of ICIs to primarily focus on this type of combined immunotherapy.

Table 2. Factors influencing patient prognosis or efficacy for treating hepatocellular carcinoma with immune checkpoint inhibitors.

Agents	Study design	Number of cases	Prognostic and predictive factors	Out-come	analysis	HR [95%CI]	P-value	Author (reference no)
Anti-PD-(L)1-based immuno-therapy	Meta-analyses of 3 phase 3 trials: Checkmate 459 (Nivo vs Sora), IMbrave 150 (Ate/Bev vs Sora), KEYNOTE-240 (Pembro vs PBO) Retrospective (ICI single arm)	ICI 985 Nivo 371 Pembro 278 Ate/Bev 336 Control 672 Sora 372+165 Placebo 135 exploratory 130 validation 118	[HBV] [HCV] [NAFLD]	OS OS OS	univariate univariate multivariate	0.64 [0.49-0.83] 0.68 [0.48-0.97] 2.6. [1.2-5.6]	0.0008 0.04 0.017	Pfister D et. al. [75]
Ate/Bev Lenva (Sora)	retrospective	Non-viral cohort Ate/Bev 190 Len 569 NAFLD/NASH cohort Ate/Bev 82 Len 254	[Lenvatinib] [Lenvatinib]	OS PFS OS PFS	multivariate multivariate multivariate multivariate	0.65 [0.44-0.95] 0.67 [0.51-0.86] 0.46 [0.26-0.84] 0.55 [0.38-0.82]	0.0268 0.035 0.011 0.031	Rimini M et. al. [77]
Anti-PD-(L)1 mono-therapy	retrospective, single arm	18	[hyperintensity tumor (RER* ≥ 0.9) on EOB-MRI]	PFS	multivariate	7.78 [1.59–38.1]	0.011	Aoki T et. al. [82]
Ate/Bev	retrospective validation based on multiomics study, single arm	Non-viral HCC 30	[Steatotic HCC]	PFS	univariate		<0.05	Murai H et.al. [85]

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Ate/Bev	retrospective, separate single arm (not vs Len)	Ate/Bev 35	[hetorogenous tumor on EOB-MRI]	PFS	univariate		0.007	Sasaki R et.al. [86]
Lenva			[hyperintensity tumor (RER‡ ≥ 0.9) on EOB-MRI]	PFS	univariate		0.012	
		Len 33	(no significant factor)					
Anti-PD-(L)1-based immuno-therapy	retrospective, single arm	24	[20 gene inflamed signature] (CCL5, CD2, CD3D, CD48, CD52, CD53, CXCL9, CXCR4, FYB, GZMA, GZMB, GZMK, IGHG1, IGHG3, LAPTM5, LCP2, PTPRC, SLA, TRAC, TRBC2)	PR	Wilcoxon rank-sum		0.047	Montironi C et.al. [91]
Anti-PD-(L)1-based immuno-therapy	retrospective, separate single arm (not vs Sora)	Anti-PD-(L)1-based immuno-therapy: training 190 (anti-PD-(L)1 mono 110, Ate/Bev 75, Others 5) validation 102 (anti-PD-(L)1 mono 68, Ate/Bev 25, Anti-PD-(L)1 + TKI 7, Others 2)	[Child-Pugh A]	OS	multivariate	2.3 (1.5-3.4)	<0.001	Scheiner B [93]
			[ECOG PS 0]	OS	multivariate	2.1 (1.4-3.2)	<0.001	
			[AFP<100]	OS	multivariate	1.7 (1.2-2.6)	0.007	
			[CRP<1]	OS	multivariate	1.7 (1.2-2.6)	0.007	
			[CRAFITY score†]	OS	univariate		0.001	
			CRAFITY low			1		
			CRAFITY int.			2.0 [1.1-3.4]		
			CRAFITY high			3.6 [2.1-6.2]		
			[CRAFITY score†]	ORR	Chi square	-	0.001	
			[CRAFITY score†]	DCR	Chi square	-	<0.001	
Sora			[CRAFITY score†]	OS	univariate	-	0.001	
				DCR	Chi square	-	0.037	
		Sora 204	[CRAFITY score†]	OS	univariate	-	<0.001	
Ate/Bev	retrospective, single arm	297	[AFP<100]	PFS	multivariate	-	<0.001	Hatanaka T et.al. [94]
				OS	multivariate	-	0.028	
			[CRP<1]	PFS	multivariate	-	<0.001	
				OS	multivariate	-	0.032	
			[CRAFITY score†]	PFS	univariate	-	<0.001	
				OS	univariate	-		
				DCR	Chi square	-	0.029	
Ate/Bev	retrospective, single arm	40	[NLR > 3.21]	PFS	univariate	-	<0.0001	Eso Y et.al [99]
Ate/Bev	retrospective, single arm	249	[NLR > 3]	OS	multivariate	3.37 [1.02-11.08]	0.001	Tada T et.al. [100]
Ate/Bev	retrospective pooled analysis	GO30140 arm A	<Transcriptome > [ABRS[a] high]	PFS	univariate	0.51 [0.3-0.87]	0.013	Zhu AX et. al.

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Sora	of the phase 1b GO30140 (single arm) and the phase 3 trial IMbrave 150 (Ate/Bev vs Sora)	(Ate/Bev 90 single arm)	[CD274[b] high]	PFS	univariate	0.42 [0.25-0.72]	0.0011	[72]
			[Teff[c] high]	PFS	univariate	0.46 [0.27-0.78]	0.0035	
			<In situ analyses>					
			[CD8+Tcell density]	CR/PR	Student T	-	0.007	
			[CD3+Tcell density]	CR/PR	Student T	-	0.039	
			[CD3+GZMB+Tcell density]	CR/PR	Student T	-	0.044	
			[MHC1+ tumor cells]	CR/PR	Student T	-	0.0087	
		IMbrave 150 (Ate/Bev119 Sora 58)	<Transcriptome >	PFS	multivariate	0.49 [0.25-0.97]	0.041	
			[ABRS[a] high]	OS	multivariate	0.26 [0.11-0.58]	0.0012	
				PFS	multivariate	0.46 [0.25-0.86]	0.015	
			[CD274[b] high]	OS	multivariate	0.3 [0.14-0.64]	0.002	
				PFS	multivariate	0.52 [0.28-0.99]	0.047	
			[Teff[c] high]	OS	multivariate	0.24 [0.11-0.5]	0.0002	
				PFS	multivariate	0.42 [0.22-0.79]	0.007	
			[Treg[d]/Teff[c] low]	OS	multivariate	0.24 [0.11-0.54]	0.0006	
				PFS	multivariate	0.47 [0.27-0.81]	0.006	
			[GPC3 low]	OS	multivariate	0.29 [0.13-0.62]	0.002	
				PFS	multivariate	0.49 [0.28-0.87]	0.014	
			[AFP low]	OS	multivariate	0.32 [0.14-0.73]	0.007	
			<In situ >	OS	multivariate	0.29 [0.14-0.61]	0.0011	
			[CD8+Tcell high dens.]	PFS	multivariate	0.54 [0.29-1.00]	0.053	
				OS	multivariate	0.42 [0.19-0.91]	3×10^{-4}	
			<Genetic profiling>	PFS	multivariate	0.45 [0.27-0.86]	0.0086	
			[CTNNB1 WT]	OS	multivariate	0.38 [0.16-0.89]	7.8×10^{-5}	
				PFS	multivariate	0.61 [0.33-1.10]	0.047	
			[TERT Mut]					
Ate/Bev	retrospective, single arm	34	[high plasma IL-6]	PFS OS	univariate multivariate univariate	- 2.785 [1.216-6.38] -	<0.05 0.01 <0.05	Myojin Y et.al. [103]
Ate/Bev Len	retrospective, separate single arm (not vs Len)	Ate/Bev 24 Len 15	[High-level CD8+ TILs] (no significant factor)	PFS ORR DCR	univariate Chi square Chi square	- - -	0.041 0.012 0.031	Kuwano A et.al. [104]

HR, hazard ratio; CI, confidence interval; PFS, progression-free survival; OS, overall survival; PD-1, Programmed death receptor 1; PD-L1, Programmed cell Death ligand 1; HBV, hepatitis B virus; HCV, hepatitis C virus; NAFLD,nonalcoholic fatty liver disease; NASH, Nonalcoholic steatohepatitis; RER, relative enhancement ratio; EOB-MRI, gadolinium ethoxybenzyl diethylenetriaminepentaacetic acid-enhanced magnetic resonance imaging; HCC, hepatocelluar carcinoma; PR, partial response; ECOG, Eastern Cooperative Oncology Group; AFP, α-fetoprotein; CRP, c-reactive protein; ORR, overall response rate; DCR, disease control

rate;NLR, neutrophil to lymphocyte ratio; CR, complete response; PR, partial response. Note: In the 'Statistical analysis' section, 'univariate' typically refers to the Kaplan-Meier method and log-rank test, while the inclusion of HR indicates the use of Cox regression. Additionally, 'multivariate' typically refers to the utilization of the multivariate Cox regression model. ‡: (nodule SI/parenchyma SI on hepatobiliary phase images)/(nodule SI/parenchyma SI on precontract images) SI: signal intensity. †: CRAFITY-low: AFP<100 & CRP<1, intermediate: AFP≥100 ng/ml or CRP≥1 mg/dl, high: AFP ≥ 100 ng/mL & CRP ≥ 1 mg/dL. a: ABRS, atezolizumab + bevacizumab response signature (including CXCR2P1, ICOS, TIMD4, CTLA4, PAX5, KLRC3, FCRL3, AIM2, GBP5, and CCL4). b: CD274, PD-L1 mRNA. c: Teff, T effector (including CXCL9, PRF1, and GZMB). d: Treg, T regulatory (including CCR8, BATF, CTSC, TNFRSF4, FOXP3, TNFRSF18, IKZF2, and IL2RA).

4.1. Known Predictive Markers for the Efficacy of Single-Agent ICI and Combined Immunotherapies for HCC: PD-L1 Expression, Tumor Mutation Burden (TMB), and Microsatellite Instability (MSI)

The discovery of immune checkpoint proteins, such as PD-1/PD-L1 and CTLA-4, represents a significant breakthrough in the cancer immunotherapy field [68,69]. Currently, anti-PD-1 antibodies, anti-PD-L1 antibodies, and anti-CTLA-4 antibodies are used to treat HCC, including in combination therapies. For other types of cancer, PD-L1 expression, TMB, and MSI have been reported as biomarkers for predicting the therapeutic effects of these ICIs [70]. However, when considering the practicality of these as biomarkers, the frequency of PD-L1 expression, TMB-High, and MSI-High becomes an issue. According to a large cohort study by Ang et al., the incidence of MSI-High in HCC was extremely limited, with only one case among 542. Additionally, only six cases (0.8%) among 755 cases had a TMB of 20 mutations/Mb or more [71].

Zhu et al. conducted comprehensive analyses of transcriptomics, genomics, and IHC staining of patient samples collected in Phase Ib GO30140 trials and Phase III IMbrave150 trials to explore biomarkers for atezolizumab and bevacizumab combination therapy [72]. Either whole exome sequencing (WES) or FoundationOne panel profiling was performed to evaluate TMB, resulting in median TMBs of 5.6 mutations/Mb and 4.4 mutations/Mb, respectively. TMB was categorized as low, medium, or high, and its associations with response rates and survival times were verified. However, the relationship between TMB and response rate or survival benefit did not coincide in the GO30140 trial arm A and IMbrave 150 [72].

PD-L1 expression merits further investigation. In the phase I/II CheckMate 040 trial of nivolumab monotherapy for advanced HCC, PD-L1 expression in tumor tissues was examined in 174 out of 214 cases in the dose-expansion phase. Of these, 34 cases (20%) showed PD-L1 expression ≥ 1% in tumor cells via IHC, and these cases demonstrated an ORR of 9/34 (26%; 95% CI 13–44). However, even in 140 cases with PD-L1 <1 %, an OR was observed in 26/140 cases (19%; 95% CI 13–26), suggesting that therapeutic responses were observed regardless of PD-L1 expression status [63].

In the phase III Checkmate459 trial of nivolumab vs. sorafenib, PD-L1 expression ≥ 1% in tumor cells was found in 71 of 366 cases (19%) in the nivolumab group and in 64 of 362 cases (18%) in the sorafenib group. In patients who were administered nivolumab, a higher ORR was indicated if PD-L1 ≥ 1% than if PD-L1 < 1% (PD-L1 ≥ 1% ORR 20/71 (28%; 18–40); PD-L1 < 1% ORR 36/295 (12%; 9–17)). However, in the sorafenib group, there was no difference in ORR whether PD-L1 ≥ 1% or PD-L1 < 1% (PD-L1 ≥ 1% ORR 6/64 (9%; 4–19); PD-L1 < 1% ORR 20/300 (7%; 4–10)). A comparison between the nivolumab group's PD-L1 ≥ 1% and the sorafenib group's PD-L1 ≥ 1% showed a trend that favored nivolumab, with a median OS of 16.1 months (95% CI 8.4–22.3) vs. 8.6 months (95% CI 5.7–16.3), but the difference was not statistically significant (HR 0.80 (0.54-1.19)) [64].

In the phase II Keynote224 trial of pembrolizumab monotherapy, the conventional positive cell rate of PD-L1 in tumor cells (tumor proportion score (TPS)) and the combined positive score (combined positive score (CPS)) were calculated by dividing the number of PD-L1 positive cells in tumor cells and immune cells by the total number of surviving tumor cells and multiplying by 100 [73]. Of 52 cases, 22 cases (42%) were CPS positive and only seven cases (13%) were TPS positive. Significant differences were observed in the response rates and PFS between CPS positive and negative cases, but not between TPS positive and negative cases [73].

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According to the comprehensive analysis by Zhu et al., patients with high CD274 (PD-L1 mRNA) expression showed a longer PFS with the atezolizumab-bevacizumab combination therapy than those with low expression. However, IHC data for PD-L1 protein levels indicated that there was only a potential correlation between PD-L1 expression and response [72].

As these results suggest, PD-L1 expression is somewhat related to the efficacy of anti-PD-1/PD-L1 immunotherapy in HCC and could potentially serve as a biomarker for predicting therapeutic effects. However, there are some uncertainties, as it is difficult to definitively make these predictions at the protein level, it is not yet determined whether to use TPS or CPS scoring, and there is an issue of heterogeneity associated with IHC staining assays [74].

4.2. NASH as a Background Liver Disease

One potential biomarker that could predict the lack of efficacy of ICI monotherapy is NASH/NAFLD as a background liver disease. Pfister et al. suggested that in a mouse model of NASH-induced HCC, CD8+/PD-1+ T cells could promote the progression of NASH. Administration of ICIs could release the brakes on these NASH-promoting cells, resulting in a potential exacerbation of NASH and increased HCC occurrence [75]. The authors conducted a meta-analysis of the cohorts from three phase III trials where ICIs were administered, namely Checkmate 459, IMbrave 150, and KEYNOTE-240. This analysis showed that while the ICI treatment significantly prolonged OS compared with the control in HBV-related and HCV-related HCC cases, prognosis did not improve in non-viral HCC cases. Furthermore, in two separate retrospective cohorts treated with anti-PD-1 or anti-PD-L1 antibodies, HCC cases caused by NAFLD showed reduced OS compared with those with other etiologies [75]. As demonstrated in this study, in a subgroup analysis of the phase III IMbrave 150 trial of atezolizumab-bevacizumab combination therapy and sorafenib, non-viral HCC including NASH did not show superiority, with a median OS of 17.0 months in the atezolizumab-bevacizumab combination group compared with 18.1 months in the sorafenib group [76].

Moreover, in a multicenter study involving 36 facilities in four countries (Italy, Japan, South Korea, and the UK), a retrospective analysis of 759 cases of advanced non-viral HCC revealed that when lenvatinib and atezolizumab + bevacizumab treatments were compared, lenvatinib showed significantly better results for OS and PFS rates in non-viral HCC overall. When non-viral HCC was divided into NAFLD/NASH and non-NAFLD/NASH, lenvatinib treatment was associated with a significant survival benefit compared with atezolizumab + bevacizumab in patients with NAFLD/NASH HCC [77]. These studies suggest the potential for the background liver disease in HCC to serve as a biomarker when deciding if ICI therapy is appropriate.

4.3. Wnt/β-Catenin Mutations as a Biomarker and MRI Findings as Imaging Biomarkers

Spranger et al. reported that the presence of Wnt/β-catenin mutations in melanoma results in the exclusion of T cell infiltration and resistance to ICIs [78]. Using The Cancer Genome Atlas (TCGA), Luke et al. demonstrated that tumors lacking the genetic expression signature of T cell-mediated inflammation, including 31 types of solid cancers like melanoma, show activated Wnt/β-catenin signaling [79]. Furthermore, Harding et al. conducted a genomic analysis of 127 HCC tumor tissues, reporting that Wnt/β-catenin mutations were found in 45% of cases. While the presence or absence of these mutations did not affect PFS with sorafenib treatment, their presence significantly shortened PFS with ICI treatment (2.0 vs. 7.4 months, $P < 0.0001$) [80]. In addition, in a comprehensive study by Zhu et al., patients with a wild-type CTNNBI genotype in the IMbrave150 trial showed a greater therapeutic effect with atezolizumab + bevacizumab compared with sorafenib treatment, but no significant difference was observed between the treatments in cases with CTNNBI mutations [72].

Ueno et al. focused on the differences in HCC findings in the hepatobiliary phase of gadolinium ethoxybenzyl diethylenetriamine pentaacetic acid (Gd-EOB-DTPA) enhanced MRI, comprehensively examined the transporter of Gd-EOB-DTPA, and analyzed the molecular regulatory mechanism. In clinical samples, it was demonstrated that high expression levels of OATP1B3 were strongly correlated with greater enhancement in the hepatobiliary phase of Gd-EOB-DTPA enhanced MRI.

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Additionally, activated Wnt/β-catenin signaling is closely associated with OATP1B3 expression in HCC cell cultures [81].

Aoki et al. analyzed 18 HCC cases that had received anti-PD-1 or anti-PD-L1 monotherapy and had Gd-EOB-DTPA enhanced MRI taken before treatment. As a result, in cases with high signal nodules in the hepatobiliary phase (n = 8), the median PFS was 2.7 months, while in cases with low signal nodules (n = 10), it was 5.8 months (P = 0.007). There was also a significant difference in the period until tumor enlargement, indicating that the hepatobiliary phase of Gd-EOB-DTPA enhanced MRI is a promising imaging biomarker for predicting the therapeutic effect of anti PD-1/PD-L1 monotherapy [82].

Moreover, it should be noted that multiple studies have reported that the efficacy of lenvatinib treatment for HCC is not affected by the signal intensity of the hepatobiliary phase of EOB-MRI [83,84].

Murai et al. focused on the increasingly prevalent non-viral HCC cases. They extracted the genomic DNA and total RNA from tumor tissues for profiling, then compared them with the pathological findings to identify sensitivity to immunotherapy. Steatotic HCC accounted for 23% of non-viral HCC cases, which showed an immune-rich, yet immune-exhausted, tumor immune microenvironment characterized by T cell exhaustion, infiltration of M2 macrophages and cancer-associated fibroblasts (CAFs), high expression of immune PD-L1, and activation of TGF-β signaling. Histological fatty deposition of resected HCC tissue and Fat Fraction Corrected for Spectral Complexity and Inhomogeneities (FFCSI) measured by MRI were strongly correlated. When 30 HCC patients evaluated by MRI before atezolizumab-bevacizumab combination therapy were retrospectively reviewed, a significantly longer PFS was confirmed in patients with steatotic HCC [85].

Whether MRI findings are useful as imaging biomarkers for predicting the efficacy of systemic therapy for HCC remains to be demonstrated with prospective validation results. However, as the number of cohorts of systemic therapy using ICIs, such as atezolizumab-bevacizumab combination therapy, increases in clinical practice, we believe a consensus will form.

4.4. Problems with Wnt/β-catenin mutations as a biomarker and MRI findings as imaging biomarkers

With the combination therapy of atezolizumab and bevacizumab, Sasaki et al. reported that HCC patients with high-intensity EOB-MRI hepatobiliary phase suggesting Wnt/β-catenin signal activation had a shorter PFS than the low-intensity HCC patients in the atezolizumab + bevacizumab group. This MRI finding was not associated with the treatment effect of lenvatinib [86]. However, in a study by Kuwano et al. using not EOB-MRI, but pretreatment tumor biopsy, there was no significant difference in the treatment effect or PFS of those receiving atezolizumab/bevacizumab combination therapy that depended on the presence or absence of Wnt/β-catenin activation [87]. These discrepancies may be from biases resulting from each study being retrospective and having a small number of cases, but some research results may suggest otherwise.

First, previous research results have indicated that there may be a discrepancy between EOB-MRI hepatocellular phase uptake findings and Wnt/β-catenin mutations. The transcription factor HNF4α, which plays a role in maintaining mature hepatocyte function, decreases in dedifferentiated HCC. A decrease in HNF4α during HCC dedifferentiation lead to decreased expression of OATP1B3 regardless of Wnt/β-catenin mutations. This then results in a loss of gadoxetic acid uptake in the hepatocyte phase, which may cause a mismatch [88,89].

Another question is if the presence of Wnt/β-catenin mutations in HCC always results in a suppressed immune response. Sia et al. reported that about 25% of HCC cases are a subtype of immune class characterized by immune activation, with an overexpression of adaptive immune response genes, such as CD8A, CD3E, IFNG, CXCL9, and others, in the active immune response subtype. Additionally, immunosuppressive signals, like TGF-β, and M2 macrophages are present in the exhausted immune response subtype. The authors also stated that a better response to ICI treatment is expected in this immune class [90].

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This research group further investigated the immune characteristics of HCC cases outside this immune class. As a result, they found that about 10% of HCC cases have an immune-like class characterized by high interferon (IFN) signaling, cytokines, and a diverse T cell repertoire, despite significant activation of Wnt/β-catenin signaling by CTNNB1 mutation. This led them to classify HCC into an inflamed class, which includes the immune class and the immune-like class, and the other non-inflamed class. They designed an 'inflamed signature' consisting of 20 genes that can accurately indicate the inflamed class and confirmed that there was a significant overexpression of this signature in a group of patients who showed PR (partial response) with ICI treatment in an external cohort compared with a group of patients who showed SD (stable disease) and PD (progressive disease) [91].

When evaluating the tumor microenvironment, immunostaining in the inflamed class showed an enrichment of intratumoral CD8+ T cells (CD8 ≥ 1%, 58% vs. 30%, P=0.08) and PD-L1 (PD-L1 ≥ 1%, 21% vs. 4%, P=0.19) compared with the non-inflamed class. Additionally, analysis using CIBERSORT, which estimates the presence and ratios of immune cell subsets within tissues from gene expression data, showed a significantly higher proportion of CD8+ T cells (P=3.51×10-7) and M1 macrophages (P=1.82×10-4). However, in the immune-like class, M2 macrophages were significantly excluded (P=1.78×10-6) [91].

Furthermore, the authors created a 13-protein signature for identifying the inflamed class using a cohort with blood samples, as liquid biopsy-based biomarkers. They suggested that in treating HCC, it is worth considering whether to distinguish between the inflamed and non-inflamed classes using the 20-gene signature in tumor tissues or the liquid biopsy-based signature [91].

Interestingly, a study using a dataset of over 9,000 solid cancer cases across 31 types from TCGA found that activation of the Wnt/β-catenin pathway is often associated with reduced T cell infiltration in most human cancers. A significant inverse correlation was observed between β-catenin protein levels and T cell inflammatory gene expression in 177 HCC cases [92]. According to this study, the degree of the inverse correlation between β-catenin protein levels and T cell inflammatory gene expression varied by cancer type. Furthermore, in certain types of cancer, such as colorectal and rectal cancers, examples where T cell inflammation and activation of the Wnt/β-catenin pathway coexist are not rare [92]. This suggests that while activation of the Wnt/β-catenin pathway often hinders T cell inflammation in human cancers, it is not always the case. However, it seems that subclasses, like the immune-like class, have not been proposed for other types of cancers.

Several studies have indicated the possibility that the hepatocyte phase of EOB-MRI may not match with Wnt/β-catenin mutations, and that there may be HCC cases of the immune-like class that do not suppress immune responses against the tumor, even with Wnt/β-catenin mutations. There may be potential in using findings from the hepatobiliary phase of EOB-MRI as biomarkers to predict the therapeutic effect of ICIs or the combination therapy of atezolizumab and bevacizumab. However, these findings do not necessarily indicate the presence or absence of Wnt/β-catenin mutations. When using the presence or absence of Wnt/β-catenin mutations as biomarkers to predict the therapeutic effect of ICIs, it is necessary to consider the existence of the immune-like class.

4.5. Blood Sample Biomarkers for Predicting the Therapeutic Effect of ICI Therapy: CRAFITY Score and NLR

Scheiner et al. created a training set of 190 cases and a validation set of 102 cases from a database of HCC cases in Europe that had received PD-L1/PD-1-based immunotherapy. Seventy-five cases (40%) of the training set and 25 cases (25%) of the validation set were patients who had received atezolizumab and bevacizumab combination therapy. In the training set, the investigated baseline parameters were etiology, whether immunotherapy was primary or after other treatments, Childs-Pugh class, ECOG performance status, radiological criteria, including the presence of major vessel invasion and extrahepatic metastasis, and serum AFP and C-reactive protein (CRP) levels. Serum AFP < 100 vs. ≥ 100 ng/mL and CRP < 1 vs. ≥ 1 mg/dL were identified as independent prognostic factors in the multivariate analysis, and the CRAFITY score was developed using this [93].

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Patients with a score of 0 (CRAFITY-low: AFP < 100 ng/mL and CRP < 1 mg/dL) had the longest OS, followed by those with a score of 1 (CRAFITY-intermediate: either AFP ≥ 100 ng/mL or CRP ≥ 1 mg/dL) and those with a score of 2 (CRAFITY-high: both AFP ≥ 100 ng/mL and CRP ≥ 1 mg/dL). Similarly, the best treatment effect was seen in patients with a low CRAFITY score. This study also validated a cohort of 204 cases of sorafenib administration. The CRAFITY score was associated with the survival of the individuals, but not with the therapeutic effect [93]. The C-statistics, one of the statistical indicators to evaluate the performance and predictive ability of the model in the CRAFITY score, was 0.62 for both the derivation and validation cohorts. Although not highly accurate, it is very outstanding in its ease of use in routine practice and may be useful for predicting responses to ICI.

In a multi-institutional retrospective study in Japan, the CRAFITY score of 297 patients who received atezolizumab and bevacizumab combination therapy was analyzed. The median PFS in the CRAFITY score 0, 1, and 2 groups was 11.8 months, 6.5 months, and 3.2 months, respectively ($p < 0.001$). The median OS in patients with CRAFITY score 0, 1 and 2 was not reached, 14.3 months, and 11.6 months, respectively. This study showed the CRAFITY score could be useful for predicting therapeutic outcomes [94].

The pre-treatment NLR reflects the inflammatory response to cancer and is reportedly associated with patient prognosis and response to ICI treatment in various tumors [95–98]. Eso et al. analyzed the course of 40 HCC patients who received atezolizumab and bevacizumab combination therapy and found that the NLR value was significantly lower in the complete response (CR), PR and SD group than in the PD group (2.47 vs. 4.48, P = 0.013). Using the optimal NLR cut-off value (3.21) determined by receiver operating characteristic curve analysis for predicting response, they also found that patients with NLR ≤ 3.21 showed significantly better PFS than patients with NLR > 3.21 [99].

A similar examination was conducted in a multi-institutional joint study in Japan, and the cumulative OS rate was significantly different between patients with low NLR (< 3.0) and high NLR (≥3.0) (P = 0.001). Conversely, there was no difference in cumulative PFS or response between patients with low and high NLR values. In Cox proportional hazards modeling analysis using inverse probability weighting, having an NLR of at least 3.0 was found to be significantly associated with OS [100].

Because the CRAFITY score and NLR are parameters that can be easily obtained from blood samples, it is necessary to rigorously validate whether they should be used as biomarkers in routine practice.

4.6. Biomarkers Predicting the Therapeutic Effect of Atezolizumab and Bevacizumab Combination Therapy

As will be discussed below, the results of the HIMALAYA trial have made it possible to administer a combination of durvalumab and tremelimumab as a first-line treatment. However, comparisons of treatment outcomes have indicated that the first-line treatment of choice for unresectable HCC cases is currently thought to be atezolizumab and bevacizumab combination therapy [101,102].

Myojin et al. measured the levels of 34 baseline plasma proteins in patients with advanced HCC who received atezolizumab + bevacizumab therapy, finding that plasma IL-6 levels were a significant predictor of non-response to this therapy. They confirmed that the PFS and OS were significantly shorter in the high IL-6 group than in the low IL-6 group [103].

As mentioned previously, Zhu et al. used transcriptome analysis to derive an atezolizumab + bevacizumab response signature (ABRS) comprising 10 genes associated with a response to atezolizumab + bevacizumab (defined as CR or PR). High expression of the ABRS, as well as the existing immune gene CD274 (PD-L1 mRNA) or the Teff sign (CXCL9, PRFI, and GZMB), was associated with longer PFS in patients treated with atezolizumab + bevacizumab. The Treg signature (CCR8, BATF, CTSC, TNFRSF4, FOXP3, TNFRSF18, IKZF2, and IL2RA) was also related to improved PFS and OS when the Treg/Teff signature ratio was low in IMbrave150, which compared atezolizumab + bevacizumab with sorafenib treatment [72].

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Multiplex IHC analysis in this study showed that in the GO30140 cohort A, responding patients (CR/PR) had a higher density of infiltrating CD8+ T cells, CD3+ T cells, and GZMB+/CD3+ T cells in tumor areas than non-responders (SD/PD). Furthermore, the density of tumor-infiltrating CD8+ T cells in IMbrave 150 baseline tumor samples was analyzed. Patients with a high density of tumor-infiltrating CD8+ T cells (defined by the split median) had significantly longer OS when treated with atezolizumab + bevacizumab combination therapy compared with those treated with sorafenib [72]. These studies highlight an important point: the state of T cell immunity in the tumor microenvironment before treatment ultimately influences the treatment effect of atezolizumab + bevacizumab combination therapy for HCC.

Kuwano et al. examined the relationship between tumor infiltration of CD8+ T cells detected by IHC staining of liver tumor biopsies before treatment initiation and the therapeutic effect of drug therapy. In cases with a high level of CD8+ T cell tumor infiltration, the PFS of patients treated with atezolizumab + bevacizumab combination therapy was significantly extended and the response rate was also significantly improved compared with cases with low levels. However, in patients receiving lenvatinib, there was no association between CD8+ T cell tumor infiltration and the response rate or PFS [104]. Although this study included only a limited number of patients, it suggests that evaluating CD8+ T cell tumor infiltration alone, without any transcriptomics analysis or genomic profiling, may serve as a useful biomarker for predicting the treatment method choice and therapeutic response to drug therapy in HCC.

4.7. Biomarkers for Durvalumab and Tremelimumab Combination Therapy

The combination therapy of the anti-PD-L1 antibody durvalumab with the anti-CTLA-4 antibody tremelimumab for unresectable HCC cases has surpassed the control drug sorafenib in the HIMALAYA trial and has been approved as a first-line therapy [11]. Among the factors studied as potential biomarkers in the HIMALAYA trial, the only results currently available relate to the PD-L1 status of the tumor prior to therapy. According to the subgroup analysis, there was no difference in benefits of a combination of the two ICIs compared with sorafenib, regardless of whether PD-L1 expression was positive or negative [11].

Another interesting finding was that the combination therapy showed clear advantages over sorafenib treatment in cases of HBV-related and non-viral HCC, but not in cases of HCV-related HCC. A similar trend was observed with durvalumab monotherapy [11]. As mentioned in the sorafenib section of this review, this may be because sorafenib has greater benefits for HCV-related HCC [15]. Nevertheless, there is no clear biomarker candidate for the durvalumab and tremelimumab combination therapy. The CRAFITY score and NLR can be easily validated, but as of now, no reports have confirmed their effectiveness with this treatment.

5. Conclusions and Future Directions

In this review, we have discussed the published research findings on biomarkers for predicting the therapeutic effects of drugs available for unresectable HCC tumors. As TKIs will continue to be used as secondary therapies, the search for biomarkers must continue. Among the factors mentioned, hepatic function, underlying hepatic disease, and the NLR may serve as vague indications of utility, but they are far from being decisive in drug selection. Because it is considered difficult to carry out prospective trials of existing TKIs in the future, we believe it would be worthwhile to proactively investigate if biomarker candidates like DKK-1, ST6GAL1, and regorafenib hold value, as they have been indicated in several studies. We encourage this investigation within existing cohorts that have retained blood samples.

For the current standard treatment of atezolizumab and bevacizumab combination therapy, routine examinations such as underlying hepatic disease, CRAFITY score, and the NLR seem to provide some guidance. Furthermore, imaging diagnostics, such as the evaluation of fat deposition through EOB-MRI hepatobiliary phase or FFCSI and information from MRI examinations, can be useful. At present, the most important factor is believed to be an accurate assessment of the state of T cell immunity in the tumor microenvironment prior to treatment. The literature suggests that

evaluating the tumor microenvironment and immune environment is more achievable compared with the diversity and heterogeneity of tumor cells. Assessing CD8+ T cell infiltration by collecting tumor tissues can be performed relatively easily, even in general hospitals. Thus, this has a high potential to become a practical biomarker.

In general hospitals that treat HCC patients, it is not necessarily wrong to administer drug therapy for advanced HCC cases according to guidelines based only on the results of blood tests and CT scans. However, we recommend conducting treatment after assessing the state within the tumor tissue as much as possible by collecting blood and tissue samples and performing MRI image evaluations before treatment.

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