

Current state and challenges of emerging biomarkers for immunotherapy in hepatocellular carcinoma (Review)

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Abstract. Hepatocellular carcinoma (HCC) is the most prevalent form of primary liver cancer. According to the American Cancer Society, among patients diagnosed with advanced liver cancer, HCC has the sixth-highest incident rate, resulting in a poor prognosis. Surgery, radiofrequency ablation, transcatheter arterial chemoembolization, radiation, chemotherapy, targeted therapy and immunotherapy are the current treatment options available. Immunotherapy, which has emerged as an innovative treatment strategy over the past decade, is serving a vital role in the treatment of advanced liver cancer. Since only a small number of individuals can benefit from immunotherapy, biomarkers are required to help clinicians identify the target populations for this precision medicine. These biomarkers, such as PD-1/PD-L1, tumor mutational burden and circulating tumor DNA, can be used to investigate interactions between immune checkpoint inhibitors and tumors. The present review summarizes information on the currently available biomarkers used for immunotherapy and the challenges that are present.

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1. Introduction

According to the 2020 global liver cancer epidemiology, liver cancer is responsible for 4.69% of all cases of cancer and 8.34% of all mortalities from cancer (1,2). Hepatocellular carcinoma (HCC) pathogenesis has been associated with infection by hepatitis B virus (HBV) and hepatitis C virus (HCV), alcohol abuse, non-alcoholic steatohepatitis, cirrhosis, and a family history of HCC, with cirrhosis caused by HBV being important as it generates 60% of all cases in China (3). Although surgical resection, radiofrequency ablation, transcatheter arterial chemoembolization (TACE), radiotherapy and chemotherapy are used as potentially curative treatments, the prognosis remains poor for patients with advanced (stage 2-4) disease (3,4). The emergence of cancer immunotherapies using immune checkpoint inhibitors (ICIs) has begun a new era of anti-tumor therapy during the past decade (5).

ICIs inhibit the activity of immune checkpoint proteins, such as PD-1, PD-L1 and CTLA-4, which restrict the immune response against tumors, thus reactivating antitumor activity (6). This immunotherapy has demonstrated promising results in patients with advanced, inoperable liver cancer and those undergoing radiofrequency ablation. For example, the IMbrave150 phase III trial demonstrated reductions in both tumor progression and mortality with the combined use of two ICIs, Atezolizumab and Bevacizumab, leading to Food and Drug Administration (FDA) approval for this drug combination as a first-line treatment for patients with unresectable or metastatic HCC (7). Additionally, the CheckMate040 and KEYNOTE-224 trials established Nivolumab and Pembrolizumab as second-line immunotherapies for liver cancer, although subsequent trials did not observe an improvement in overall survival (OS) (8). Details of the current immunotherapy clinical trials are provided in Table I. However, HCC is a heterogeneous disease with multiple immunological features and thus, despite encouraging results on specific forms of HCC, the use of immunotherapy does not guarantee clinical benefit for all patients with HCC (9). Data from randomized controlled trials indicate that only 10-30% of patients with advanced HCC who undergo immunotherapy achieve a complete response (CR) or partial response (PR) (7,10-13). A major contributing factor to this is the paucity of markers for the early diagnosis and treatment of HCC. The identification and application of predictive biomarkers that can accurately

distinguish patients that would benefit from immunotherapy could enable the use of precision treatment in HCC immunotherapy, allowing the proper allocation of medical resources and avoiding the exposure of non-responsive patients to treatment toxicity. Therefore, there is an urgent need for predictive markers, whether positive or negative prognostic markers, to screen individuals for immunotherapy suitability. The present review summarizes the currently known biomarkers for immunotherapy, as presented in Fig. 1.

2. Host-associated biomarkers

Hepatitis. HCC progression is known to be associated with HBV or HCV infection and liver cirrhosis. However, evidence from the CheckMate 040 and KEYNOTE-224 trials indicated that viral load or immune responses to HBV/HCV may not necessarily influence T cell activation and subsequent antitumor activity (14,15). Furthermore, the results of a meta-analysis revealed that neither HBV nor HCV affected the tumor immune microenvironment, and the presence or absence of viral infection was not an effective criterion for the selection of patients for programmed death 1 (PD-1)/programmed death-ligand 1 (PD-L1) immunotherapy (16).

Obesity. Obesity and being overweight are considered to be risk factors for numerous diseases, including cancer (17). Obesity caused by a high-fat diet impairs CD8⁺ T cell infiltration and function, which alters the immune microenvironment in mice and enhances tumor growth (18). In contrast, another study revealed that patients with advanced HCC that had higher body mass indices (BMI; >25) appeared to have an improved prognosis following immunotherapy (19).

α -fetoprotein (AFP). In clinical practice, the serum AFP level represents a primary indicator used in diagnosis and for monitoring the effectiveness of liver cancer treatment (20). The AFP levels are increased in ~two-thirds of patients with HCC (21). The expression levels of certain immune checkpoint proteins, such as SIGLEC15, CTLA4, CD274, PDCD1LG2, PDCD1, TIGIT, LAG3 and HAVCR2, have been revealed to differ with regards to the AFP level (22). It has been suggested that AFP could be used as a prognostic biomarker for HCC immunotherapy. For example, Spahn *et al* (23) observed that baseline AFP concentrations <400 $\mu\text{g l}^{-1}$ before the start of treatment were associated with increased rates of PR or CR and reduced rates of progressive disease (PD). However, in the CheckMate 459 trial, patients with high baseline AFP levels (>400 ng/ml) had an increased overall survival (OS) (11). The objective response rate (ORR) was revealed to be positively associated with the early stages of AFP reduction therapy and PD-1 blockade, while progression-free survival (PFS) and OS were also increased (2,24). Therefore, the combination of AFP with other serum markers deserves further investigation to improve diagnostic accuracy. For example, previous studies have indicated that the C-reactive protein (CRP) and AFP in immunotherapy (CRAFITY) score, which combines CRP with AFP, can be used to predict treatment outcomes and treatment-associated adverse events in patients with HCC undergoing immunotherapy (25,26). However, there is still

disagreement over whether AFP can serve as a prognostic biomarker for immunotherapy (25,27,28).

Blood inflammatory markers. Blood inflammatory biomarkers are both affordable and useful for the early identification of disease. It has been suggested that a neutrophil-lymphocyte ratio (NLR) ≥ 5 and a platelet-lymphocyte ratio (PLRs) ≥ 300 are independent prognostic factors for OS, predicting reduced OS, PFS, ORR and an increased risk of mortality in patients receiving immunotherapy (29,30). Similarly, a multicenter study revealed that the NLR could predict PFS in patients with unresectable HCC treated with Atezolizumab plus Bevacizumab, particularly in patients with modified albumin-bilirubin grade 1 or 2a (31).

Jeon *et al* (32) revealed that the numbers of classical monocytes (such as CD14⁺CD16⁺) increased on day 7 in patients with durable clinical benefit compared with that in patients with non-durable clinical benefit. The CRP level, an indicator of inflammation, has also been revealed to have good prognostic value in lung and renal cell cancer (33-35). The baseline CRAFITY score, developed by Scheiner *et al* (26), has been demonstrated to be effective for the assessment of patients receiving immunotherapy. Specifically, the median OS was revealed to be 27.6, 11.3 and 6.4 months in the CRAFITY-high (2 points), CRAFITY-intermediate (1 point) and CRAFITY-low (0 points) groups, respectively, and the best radiological response ratio [CR/PR/stable disease (SD)/PD] is stratified based on the CRAFITY score. This use of the score was also supported by a retrospective study conducted in Japan where the OS and PFS of 297 patients that received Atezolizumab and Bevacizumab treatment were associated with AFP and CRP (25). However, the prediction model is currently only applicable to patients that receive Atezolizumab and Bevacizumab; additional validation is required for other immunotherapy medications.

Gut microbiota. According to recent studies, the gut microbiota serves an important role in the development and occurrence of liver cancer (36-38). The underlying mechanism involves the gut-liver axis and is associated with dysbiosis, intestinal permeability and bacterial metabolites. Dysbiosis and intestinal permeability make it easier for bacterial metabolites to reach the liver. Bacterial products such as lipopolysaccharides (LPS) can cause inflammation and cancer in the liver (39,40). In addition, Toll-like receptor 4 (TLR-4), which is widely distributed on the surfaces of various liver cells and has been demonstrated to mediate hepatic carcinogenesis, is the specific recognition receptor for LPS (41). In a study by Chung *et al* (36) the stools of eight antibiotic-treated patients were collected for microbiota analysis. Patients receiving Nivolumab demonstrated no alterations in the diversity and composition of their gut microbiota. However, a skewed Firmicutes/Bacteroidetes ratio and a low Prevotella/Bacteroides ratio were revealed to predict a poor immunotherapy response in patients with liver cancer, while the presence of Akkermansia species suggested a positive prognosis (36). Another study on 167 patients with hepatobiliary cancer treated with immunotherapy, revealed that a number of bacteria, such as Lachnospiraceae bacterium-GAM79, were associated with an improved OS and PFS after treatment, while other bacteria, such as *Veillonella*,

Table I. Ongoing research on immunotherapy for hepatocellular carcinoma.

Treatment	Research project	Drug	Line of therapy	Number of patients	Stage	NCT	Early result
ICI monotherapy	KEYNOTE-394	Pembrolizumab + BSC vs. BSC	Second-line	453	III	NCT03062358	mOS, 14.6 vs. 13.0 months; and ORR, 12.7 vs. 1.3%
	RATIONALE-301	Tislelizumab vs. Sorafenib	First-line	674	III	NCT03412773	mOS, 15.9 vs. 14.1 months; ORR, 14.3 vs. 5.4%; and mPFS, 2.2 vs. 3.6 months
	KEYNOTE-224	Pembrolizumab	Second-line	107	II	NCT02702414	ORR, 18.3%; mPFS, 4.9 months; and mOS, 13.2 months
ICI double	HIMALAYA	Durvalumab + Tremelimumab (STRIDE) or Durvalumab vs. Sorafenib	First-line	1,504	III	NCT03298451	mOS, 16.4 vs. 16.56 vs. 13.8 months
	CheckMate 9DW	Nivolumab + Ipilimumab vs. Sorafenib or Lenvatinib	First-line	732	III	NCT04039607	Not published
ICI + VEGF/TKI	NA	Lenvatinib + Nivolumab	First-line	50	II	NCT03841201	ORR, 28%; mPFS, 9.0 months; and mOS, 27.1 months
	LEAP-002	Lenvatinib + Pembrolizumab vs. Lenvatinib	First-line	794	III	NCT03713593	mOS, 21.2 vs. 19.0 months; and mPFS, 8.2 vs. 8.0 months
	SHR-1210-III-310	SHR-1210 (Camrelizumab) + Apatinib vs. Sorafenib	First-line	543	III	NCT03764293	mOS, 22.1 vs. 15.2 months; mPFS, 5.6 vs. 3.7 months; and ORR 25.4 vs. 5.9%
	JUPITER-10	Toripalimab + Bevacizumab vs. Sorafenib	First-line	326	III	NCT04723004	Not published

Table I. Continued.

Treatment	Research project	Drug	Line of therapy	Number of patients	Stage	NCT	Early result
ICI + locoregional therapy	EMERALD-2	Durvalumab + Bevacizumab vs. Durvalumab vs. placebo	NA	908	III	NCT03847428	Not published
	NA	Tislelizumab + Regorafenib vs. Regorafenib	First-line	125	II	NCT04183088	Not published
	IMbrave251	Atezolizumab + Lenvatinib or Atezolizumab + Sorafenib vs. Lenvatinib or Sorafenib	Second-line	554	III	NCT04770896	Not published
	GOING	Regorafenib vs. Nivolumab	Second-line	78	I/II	NCT04170556	mPFS, 6.1 vs. 6.7 months
	DEDUCTIVE	Tivozanib + Durvalumab	First-line	42	I/II	NCT03970616	mPFS, 7.3 months; and ORR, 27.8%
	EMERALD-1	Durvalumab + TACE or Durvalumab + Bevacizumab + TACE vs. TACE	NA	724	III	NCT03778957	Not published
	NA	Y-90 TARE vs. Y-90 TARE + Atezolizumab + Bevacizumab	NA	128	II	NCT04541173	Not published

BSC, best supportive care; mOS, median overall survival; ORR, objective response rate; mPFS, median progression free survival; ICI, immune checkpoint inhibitor; TKI, tyrosine kinase inhibitor; NA, not available; TACE, trans arterial chemoembolization; TARE, trans arterial radioembolization; NCT, national clinical trial.

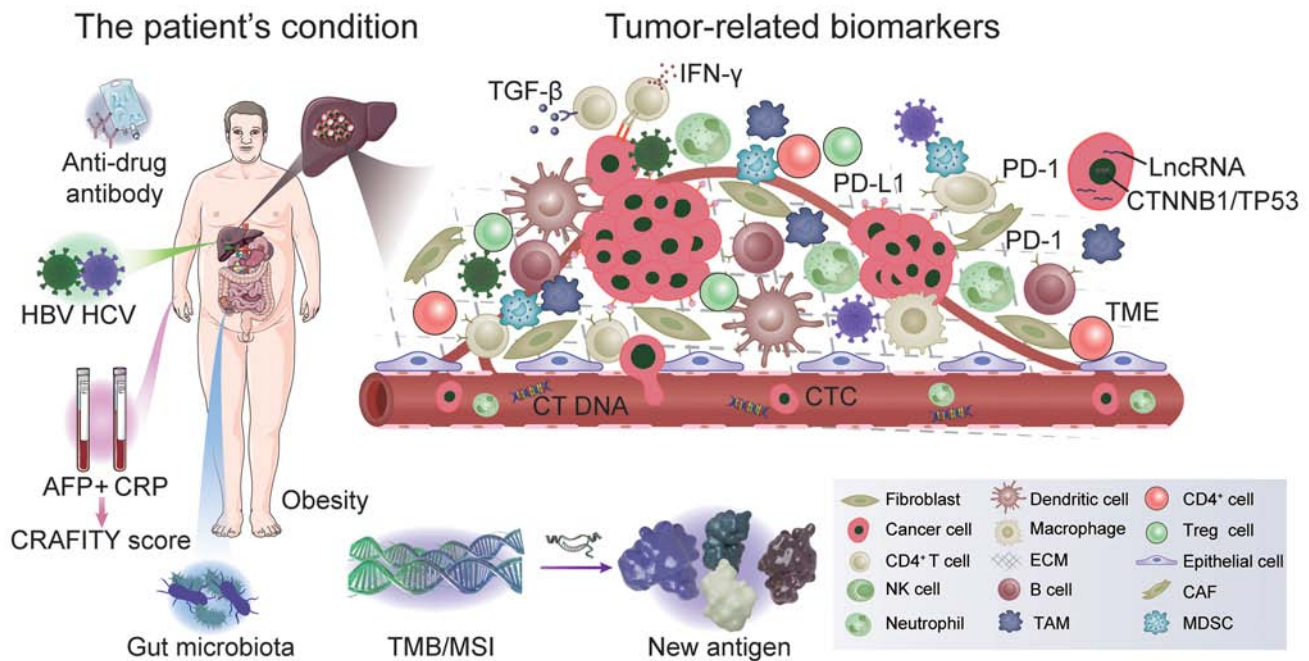


Figure 1. Potential biomarkers for predicting the response to immunotherapy in patients with liver cancer. HBV, hepatitis B virus; HCV, hepatitis C virus; TMB, tumor mutation burden; MSI, microsatellite instability; CTC, circulating tumor cell; TME, tumor microenvironment; AFP, α -fetoprotein; CRP, C-reactive protein; CRAFITY, CRP and AFP in immunotherapy; NK, natural killer; ECM, extracellular matrix; TAM, tumor-associated macrophages; Treg, regulatory T cell; ctDNA, circulation tumor DNA; CAF, cancer-associated fibroblast; MDSC, myeloid-derived suppressor cell; PD-1, programmed death 1; PD-L1, programmed death-ligand 1; lncRNA, long non-coding RNA; CTNNB1, catenin β 1; TP53, tumor protein p53.

were associated with an increased risk of immune-associated side effects (37). There is also an association between the diversity of the gut microbiota and the levels of aspartate aminotransferase and alanine aminotransferase, which reflect liver function (38). Stool samples from patients that responded to anti-PD-1 therapy contained a greater taxonomic abundance compared with those of non-responders. The characterization of the dynamic changes in the gut microbiome can be useful for an earlier prediction of anti-PD-1 treatment outcomes in HCC. With the rapid development of microbial multi-omics, analysis of the gut microbiota has potential as a predictive biomarker for liver cancer immunotherapy. It has been reported that fecal microbiota transplantation from donors that achieved CR/PR on long-term anti-PD-1 therapy to patients failed to respond to immunotherapy can increase the intra-tumoral lymphocyte infiltration (42).

Anti-drug antibodies (ADAs). ICIs may be immunogenic and recognized by the human immune system, which could lead to the induction of the humoral immunity and subsequent adverse ADA responses (43). Different monoclonal antibodies are associated with different rates of ADA development, with Atezolizumab having the highest rate (~30%) compared with others (5-10%) (44). ADAs may affect the pharmacokinetics and pharmacodynamics of therapeutic antibodies, and may even neutralize the therapeutic antibodies (45). A cohort study by Kim *et al* (46) reported that increased ADA levels at the second Atezolizumab injection (day 1 of chemotherapy treatment cycle 2) may be associated with poor clinical outcomes. Reducing Atezolizumab exposure in patients with advanced HCC, Atezolizumab and Bevacizumab administration and an established ADA level >1,000 ng/ml can accurately predict

the curative effect (46). Anti-Atezolizumab antibody-positive patients did not demonstrate a reduction in the frequency or severity of adverse events (44). However, a meta-analysis of 11 clinical trials, based on studies using Atezolizumab monotherapy or combination therapy, demonstrated that unadjusted descriptive analyses could not identify a clear association between the ADA status and the frequency or severity of adverse events. Furthermore, any ADA impact was not driven by neutralizing activity (47). The most distinctive feature of ADA assays is their lack of accurate quantification, as there is no reliable calibration reference standard for ADAs (48). Currently, there is no effective method for predicting which drugs may cause ADAs. Table II provides a brief overview of the host-associated biomarkers that are used for HCC immunotherapy.

3. Tumor-associated biomarkers

PD-1 and PD-L1. PD-1 is an immunosuppressive transmembrane protein that is expressed on the surface of cells such as T, B and myeloid cells. By binding to PD-L1, it inhibits T cell activation and proliferation, negatively stimulates T cells, blocks the T cell receptor, and negatively impacts how the immune system combats cancer (49,50). Inhibition of PD-1/PD-L1 prevents the interaction between PD-1 on T cells and PD-L1 on tumor cells, thus, restoring the T cell-mediated antitumor immune response (51). However, anti-PD-1/PD-L1 therapies are only effective in 20-40% of patients (52). Numerous studies have demonstrated that the expression level of PD-L1 on immune and tumor cells is associated with the anti-PD-1 treatment response in HCC (14,15,53,54). However, these studies varied in their detection techniques and methods

Table II. Host-associated biomarkers for HCC immunotherapy.

First author, year	Biomarker	Study design	Treatment	Patients	Outcome	Results	(Refs.)
Sangro <i>et al.</i> , 2020	AFP, PLR, NLR and HBV/HCV	Retrospective	Nivolumab	AFP <400 $\mu\text{g/l}$ (n=92); and AFP \geq 400 $\mu\text{g/l}$ (n=57)	OS	The median OS of patients with baseline AFP <400 $\mu\text{g/l}$ was increased by 3.8 months compared with that of patients with baseline AFP \geq 400 $\mu\text{g/l}$; patients with reduced NLR and PLR levels had an increased OS; and HBV/HCV replication was not associated with clinical deterioration or tumor response	(14)
Ho <i>et al.</i> , 2020	Viral etiology	Meta-analysis	ICIs	-	-	There is no effect of viral etiology on the tumor immune microenvironment in HCC, and viral status should not be used as a criterion to select patients for PD-1/PD-L1 therapy.	(16)
Akce <i>et al.</i> , 2021	BMI, NLR and sarcopenia	Retrospective	Anti-PD-1 antibody	n=57	OS	The BMI cut off value was 25; the NLR cut off value was 5.15; and sex-specific sarcopenia did not predict OS	(19)
Spahn <i>et al.</i> , 2020	AFP and TMB	Retrospective	Anti-PD-1 antibody	n=99	PFS	AFP levels \geq 400 $\mu\text{g/l}$ were associated with reduced survival rates; and there is no difference in median TMB between responders and non-responders and no correlation between TMB and PFS	(23)
Zhu <i>et al.</i> , 2022	AFP	Retrospective	Atezolizumab + Bevacizumab	n=208	PFS and OS	Patients with baseline AFP levels of >20 ng/ml, and a \geq 75% decrease or \leq 10% increase in AFP levels, measured 6 weeks after starting treatment, demonstrated an association with increased OS and PFS	(2)
Sun <i>et al.</i> , 2021	AFP and PIVKA-II levels	Retrospective	PD-1 inhibitor	n=235	ORR, PFS and OS	Early reductions (>50% after 6 weeks) in AFP and PIVKA-II levels can be predictors of the efficacy of PD-1 inhibition in patients with HCC.	(24)
Dharmapuri <i>et al.</i> , 2020	NLR and PLR	Retrospective	Nivolumab	n=103	OS	NLR <5 was associated with increased OS, and a combination of high NLR (\geq 5) and PLR (\geq 500) was associated with an eight-fold increased risk of mortality.	(29)

Table II. Continued.

First author, year	Biomarker	Study design	Treatment	Patients	Outcome	Results	(Refs.)
Muhammed <i>et al</i> , 2021	NLR and PLR	Retrospective	ICIs	n=362	PFS and OS	Patients with NLR ≥ 5 had reduced OS, PFS and ORR; and patients with PLR ≥ 300 reported reduced OS.	(30)
Scheiner <i>et al</i> , 2022	CRAFITY	Retrospective	Atezolizumab + Bevacizumab	n=102	Best radiological response (complete response, partial response, stable disease or progressive disease)	The CRAFITY score was associated with survival rates and radiological responses in patients receiving PD-(L)1 immunotherapy	(26)
Hatanaka <i>et al</i> , 2022	CRAFITY	Retrospective	Atezolizumab + Bevacizumab	0 points (n=147), 1 point (n=111) and 2 points (n=39)	PFS and OS	There were differences in the PFS and OS among CRAFITY score 0, 1 and 2 groups. The CRAFITY score is simple and can be used to predict treatment outcome.	(25)
Chung <i>et al</i> , 2021	Gut microbiome	Prospective	Nivolumab	n=8	Response rate	A skewed Firmicutes/Bacteroidetes ratio and a low Prevotella/Bacteroides ratio can serve as predictive markers for a lack of response to treatment, whereas the presence of Akkermansia species predicts a good response to treatment.	(36)
Mao <i>et al</i> , 2021	Gut microbiome	Retrospective	Anti-PD-1 antibody	n=65	PFS and OS	The gut microbiome was associated with the clinical response to anti-PD-1 immunotherapy in patients with types of hepatobiliary cancer.	(37)
Kim <i>et al</i> , 2022	ADA	Prospective	Atezolizumab + Bevacizumab	n=174	Response (complete response, partial response)	High ADA levels ($\geq 1,000$ ng/ml) may reduce Atezolizumab exposure and attenuate the anticancer efficacy of the drug.	(46)

HCC, hepatocellular carcinoma; AFP, α -fetoprotein; PLR, platelet-lymphocyte ratio; NLR, neutrophil-lymphocyte ratio; HBV/HCV, hepatitis B virus/hepatitis C virus; BMI, body mass index; TMB, tumor mutational burden; PIVKA-II, protein induced by vitamin K absence-II; CRAFITY, C-reactive protein and α -fetoprotein in immunotherapy; ADA, anti-drug antibodies; ICIs, immune checkpoint inhibitors; PD-1, programmed death 1; OS, overall survival; PFS, progression-free survival; ORR, objective response rate; PD-L1, programmed death-ligand 1.

used to measure PD-L1, so there is no universal standard for the detection and quantification of PD-L1 (55). The most commonly used methods for measuring PD-L1 are the tumor proportional score (TPS) and the combined positive score (CPS) (56).

The KEYNOTE-244 study retrospectively analyzed the association between PD-L1 expression levels and response to Pembrolizumab treatment, finding a treatment response to Pembrolizumab when PD-L1 was quantified using CPS but not when it was quantified using TPS (15). However, a study on the response to Nivolumab demonstrated different outcomes. Patients that tested positive for PD-L1 (TPS $\geq 1\%$) had an increased median OS (28.1 months) compared with those that tested negative for PD-L1 (median OS of 16.6 months) (13). Recently, a meta-analysis of nine cohort studies (seven PD-L1 and three PD-1) demonstrated that PD1/PDL-1 was a marker of poor survival rate regardless of OS, HR, CI, disease-free survival (DFS) and other evaluation methods (54). High PD-1/PD-L1 expression levels were associated with aging, multiple tumors, high α -fetoprotein levels and an advanced Barcelona Clinic liver cancer stage (14,53). In addition, PD-L1, as measured by CD274 (a PD-L1 messenger RNA) expression levels in the IMbravel50 trial, were revealed to be increased in patient with CR/PR compared with that in patients with SD/PD. Patients with high CD274 levels also demonstrated an increased PFS compared with those with low expression levels (54). However, PD-L1 expression levels are influenced by various factors. PD-L1 can be induced by IFN- γ , hypoxia or TLR-mediated pathways (57). Tumor heterogeneity and the tumor interstitium were observed to be the primary causes of inconsistent outcomes, followed by differences in detection methods (58). Thus, the value of the PD-L1 expression level as a predictive biomarker for immune checkpoint blockade therapy in HCC has been reduced.

Genetic characteristics. The CTNNB1 gene encodes the intracellular signaling transducer β -catenin, which is essential for embryonic development, cell fate determination, proliferation and migration (59). One of the key signaling pathways that control liver regeneration, homeostasis and tumorigenesis is the Wnt/ β -catenin cascade (60,61). In a mouse model of HCC, activation of this pathway promoted immune evasion and conferred resistance to anti-PD-1 therapy (62,63). Similar outcomes were observed in liver cancer. Harding *et al* (64) reported that all 10 patients with mutations in components of the Wnt- β -catenin pathway demonstrated PD and a reduced median survival rate compared with patients without mutations. This implies that the Wnt- β -catenin pathway is a marker of immunotherapy sensitivity (62). Additionally, patients with HCC with mutations in CTNNB1 were revealed to have increased OS and PFS compared with patients with no mutations in CTNNB1. Thus, CTNNB1 may serve as an independent prognostic factor in HCC following immunotherapy (65,66).

Another dysregulated signaling pathway is the transforming growth factor- β (TGF- β) pathway which is involved in inflammation, fibrogenesis and immunomodulation in the HCC microenvironment (67). Increased TGF- β signaling may lead to T cell exhaustion through the upregulation of PD-1 signaling, while inhibition of TGF- β signaling may increase the anti-tumor immunity in HCC (68). Studies using mouse

models have indicated that a combination of blocking TGF- β signaling and anti-PD-L1 antibodies could reduce TGF- β signaling, promote T cell infiltration into the tumor environment, and reshape the immune microenvironment, thus, stimulating effective anti-tumor immune responses and tumor regression (69,70).

Numerous studies are investigating cancerous genes in this era of precision medicine. Least absolute shrinkage and selection operator regression analysis of data from The Cancer Genome Atlas and International Cancer Genome Consortium dataset and the International Cancer Genome Consortium database revealed nine genes (ANP32B, BMI1, ASF1A, CDK5, BUB1, CBX3, CBX2, CDK1 and BCORL1) to be independent predictors of HCC prognosis (71). Another study identified 11 immune-associated genes, NDRG1, MAPT, FABP6, CACYBP, HSP90AA1, ISG20L2, NRAS, BRD8, OSGIN1, CD320 and PSMD14, that were used to predict immune cell infiltration and construct a prognostic index for the prediction of immunotherapy efficacy (72).

Tumor mutational burden (TMB) and microsatellite (MS) instability (MSI). The number of somatic mutations per DNA megabase (Mb), known as the TMB, is used to quantitatively evaluate the mutations carried by tumor cells (73). Greater numbers of neo-antigens, indicated by increased TMB, increases the likelihood that T cells will be recognized, which is clinically associated with improved ICI outcomes. Thus, the TMB is regarded as a reliable marker for estimating the effectiveness of immunotherapy in HCC. Data on 17 types of cancer were collected in a study by Samstein *et al* (74) confirming the initial finding that a high TMB is associated to immunotherapy effectiveness. Based in part on data from the KEYNOTE-158 study, the FDA approved the use of Pembrolizumab for solid tumors with 10 or more mutations/Mb in June 2020. However, there is not a fixed value of TMB for all types of cancer as the number of mutations defining TMB-high status varies with the type of cancer (74,75). Liver cancer has a median number of 4 mutations/Mb ($n=755$), with only 0.8% of patients having TMB-high tumors.

There are numerous studies on the role of the TMB in HCC (76,77). In a phase I clinical study, Xu *et al* (76) assessed the safety and efficacy of the combination of SHR-1210 (an anti-PD-1 antibody) and Apatinib in the treatment of patients with HCC. It was revealed that patients with a high-TMB had a worse prognosis compared with patients with a low TMB (mean, 8.53 vs. 1.44 mutations/Mb). Additionally, patients with a high-TMB had a reduced PFS with a reduction of 0.9 months compared with patients with a low TMB (76). However, only 1 patient (TMB, 15 mutations/Mb) in a fraction case series (total $n=17$) experienced a prolonged CR to ICI therapy. The TMB did not differ between responders and non-responders, highlighting the need for larger clinically annotated datasets to analyze outcome prediction (77).

Mismatch repair (MMR) in clinical practice is assessed largely by the reactions of four representative MMR-associated proteins (MLH1, MSH2, MSH6 and PMS2). One of the missing proteins is called DNA mismatch repair deficiency (dMMR) (78,79). MSI occurs during DNA replication, leading to alterations in the length or base composition of the MS, mainly as a result of dMMR. The MSI status of a tumor can

be categorized as stable (MSS), high instability (MSI-H) or low instability (80). Pembrolizumab was given FDA approval in 2017 to treat MSI-H/dMMR solid tumors that are unresectable or metastatic, have progressed after prior therapy and for which there are no adequate alternative treatment options. The first pan-cancer marker identified, MSI-H/dMMR, is now being used to direct tumor immunotherapy, and has been demonstrated to have clinical value for the treatment of tumors (81,82). Even though the incidence of the MSI-H phenotype in HCC is low at only ~2%, inflammation-mediated MMR pathway dysfunction may be to blame for the accumulation of mutations observed during hepatitis-associated tumorigenesis (78,83,84). According to several reports, Pembrolizumab treatment completely reverses MSI in patients with advanced HCC (84,85). However, a study revealed that out of 50 patients, only one (2.0%) was identified as MSI-H with high TMB, CD8⁺ lymphocyte infiltration, and low VEGF expression levels, and that patient did not experience as dramatic a response to Pembrolizumab treatment as suggested by other reports (86). MSI/dMMR is frequently used as a measure of the efficacy of immunotherapy for colorectal cancer (87). The most recent clinical study on neoadjuvant therapy for colorectal cancer included 12 patients with MSI-H/dMMR, and it revealed that all patients that finished treatment with checkpoint blockade had a clinically CR, without any reported adverse events of grade 3 or higher (88). However, another study that compared the OS of patients with resected colorectal cancer liver metastases between patients with MSS and MSI revealed that patients with MSI had a reduced OS, indicating a poor prognosis (89). The low proportion of patients with high TMB or MSI in HCC compared with gastric and colon cancer, and the sparse and contradictory information available, mainly from a small number of case reports or case series, make it impossible to determine predictive accuracy (78,86).

Tumor microenvironment (TME) components. The TME describes the area surrounding the tumor, containing various cell types, such as endothelial, immune cells and fibroblasts. Extracellular components, such as cytokines, the extracellular matrix, growth factors, hormones and peripheral blood vessels, are associated to the development and metastasis of tumors (90). In addition to these, the TME in liver cancer also contains pit cells, Kupffer cells, hepatic stellate cells, liver sinusoidal endothelial cells and hematopoietic stem cells (91). As CD8⁺ lymphocytes are the most common T cell subset, the present review focuses on them. In several tumor types, high expression levels of CD8⁺ tumor-infiltrating lymphocytes (TILs) are associated with a favorable prognosis (92). High intra-tumoral CD8⁺ TIL levels have been associated with longer OS and DFS in a meta-analysis involving a total of 3,509 patients (93). Nevertheless, according to the experimental data from the CheckMate 040 trial, increased CD3⁺ or CD8⁺ tumor-infiltrating T cells were associated with improved survival rates and treatment responses, although this association was not apparent (94). Additionally, prognosis was not revealed to be associated to macrophage markers (14). Exhausted CD8⁺ T cells also exhibit a lack of cytotoxicity, decreased release of proinflammatory cytokines, such as IL-2, IL-12, IFN- γ and TNF- α , increased expression levels of inhibitory receptors, such as PD-1 and CTLA-4, and transcriptional

and epigenetic changes (95). Additionally, compared with other types of cancer, HCC has an increased concentration of PD-1(Hi) CD8⁺ T cells that express exhaustion-associated inhibitory receptors, such as PD-1 and CTLA-4 on the surface of T cells, which is indicative of a poor prognosis (96). Immunohistochemistry (IHC) has demonstrated a strong association between an increased proportion of CD38⁺ cells and an improved response to ICIs (97). An unfavorable prognosis was revealed to be predicted by the upregulation of the LDHA, BFSP1, PPAT, NR0B1 and PFKFB4 genes, as demonstrated by a tissue microarray analysis (98). Thus, the TME can be used as a biomarker for the precise identification of patients who are sensitive to immunotherapy. However, the clinical use of TME components as biomarkers to predict the response to immunotherapy in HCC appears challenging. There is a need for the standardization and validation of test methods, test timing and test interpretation.

Circulating biomarkers. Evaluation of the treatment of patients with liver cancer should be performed throughout the treatment course, with the need for convenient, rapid and reproducible methods. It is evident that repeated multiple invasive biopsies of tumor tissue are unacceptable to patients, and the detection of circulating tumor cells (CTCs) in the blood via liquid biopsy would be more convenient for clinical use. Peripheral blood can be used to detect circulating biomarkers such as exosomes, circulating tumor DNA (ctDNA), CTCs and metabolites (99). Single- or double-stranded DNA that responds to tumor heterogeneity forms ctDNA, which is derived from tumor cells (100). According to a study by Cabel *et al* (101), synchronous changes in the ctDNA levels and the tumor size at 8 weeks after immunotherapy were predictors of DFP and OS in non-small cell lung cancer (NSCLC) and colorectal cancer. However, the plasma contains only trace amounts of ctDNA, which also fluctuates dynamically, resulting in a fluctuating detection threshold and false negatives (102). Another possible circulating biomarker is CTCs. The potential of HCC-CTCs expressing PD-L1 as prognostic and predictive biomarkers was investigated in a study by Winograd *et al* (103), which revealed that PD-L1-positive CTCs were typical of advanced HCC. Immunotherapy led to a good therapeutic response in patients with PD-L1⁺ CTCs (103). According to a different study, patients with 20% PD-L1-positive CTCs had an increased OS (median not reached vs. 8.9 months) and PFS (median 6.1 vs. 2.9 months) compared with patients with <20% PDL1-positive CTCs (76). These findings suggest that baseline ctDNA and high CTC levels might be used as predictors to select patients for immunotherapy and that dynamic changes in measured CTCs might be used as an indicator of treatment response in liver cancer, although this is still at an early stage. Further research is required on other circulating biomarkers such as extracellular vesicles and circulating RNA.

4. Combination of multiple biomarkers

There are a number of immunotherapy drugs applied in the treatment of liver cancer. Immunotherapy in combination with other anti-tumor treatments, such as TKI, VEGFR, TACE or double immunotherapy, is becoming more popular. It is challenging to identify biomarkers for the assessment

Table III. Tumor-associated biomarkers for HCC immunotherapy.

First author, year	Biomarker	Study design	Treatment	Patients	Outcome	Results	(Refs.)
Zhu <i>et al.</i> , 2018	PD-L1	Prospective	Pembrolizumab	n=104	Response (complete response, partial response)	Using TPS scores, there was not a difference in the tumor response between patients with PD-L1 <1% and PD-L1 ≥1%.	(15)
Sangro <i>et al.</i> , 2020	PD-L1 and PD-1	Retrospective	Nivolumab	PD-L1 <1% (n=159) and PD-L1 ≥1% (n=36)	OS	Tumor PD-1 and PD-L1 expression levels were associated with increased OS ($P=0.05$ and $P=0.03$, respectively).	(14)
Pinyol <i>et al.</i> , 2019	Wnt-β-catenin pathway	Retrospective	Anti-PD-1 antibody	n=10	Median survival rate	Wnt-β-catenin was a marker of immunotherapy sensitivity.	(62)
Ruiz de Galarreta <i>et al.</i> , 2019	Wnt-β-catenin pathway	Mouse model	Anti-PD-1 antibody	-	-	β-catenin activation promoted immune escape and resistance to anti-PD-1, and may represent novel biomarkers for exclusion in patients with HCC.	(63)
Wang <i>et al.</i> , 2015	Wnt-β-catenin pathway	Meta-analysis	-	β-catenin mutation (n=104) and control group (n=514)	OS	The meta-analysis revealed that the presence of β-catenin mutation, compared with the control group, was associated with an increased OS rate.	(65)
Chen <i>et al.</i> , 2021	CTNNB1	Retrospective	ICIs	-	PFS	Univariate and multivariate Cox results demonstrated that only the CTNNB1-mutant was associated with the PFS of patients with HCC in the immunotherapy cohort.	(66)
Mariathasan <i>et al.</i> , 2018	TGF-β	Mouse model	Anti-PD-1 antibody	-	-	TGF-β attenuates the tumor response to PD-L1 inhibition by contributing to the exclusion of T cells.	(69)
Xu <i>et al.</i> , 2019	TMB	Prospective	SHR-1210 (an anti-PD-1 antibody) and Apatinib	n=43	PFS	Patients with a high TMB had a worse prognosis compared with patients with a low TMB. Additionally, patients with a high TMB had a reduced PFS with a reduction of 0.9 months compared with patients with a low TMB.	(76)
Ang <i>et al.</i> , 2019	TMB and MSI	Retrospective	ICIs	n=17	Response	There were no genomic or TMB differences between patients that responded to treatment, had disease progression and had stable disease.	(77)
Kawaoka <i>et al.</i> , 2020	MSI	Retrospective	Pembrolizumab	n=2	Response	CR with OS for >10 months was achieved in 1 patient, and the other patient did not respond to immunotherapy.	(84)

Table III. Continued.

First author, year	Biomarker	Study design	Treatment	Patients	Outcome	Results	(Refs.)
Xu <i>et al</i> , 2019	CD8 ⁺ tumor-infiltrating lymphocytes	Meta-analysis	-	n=3,509	OS	The meta-analysis revealed that high levels of intra-tumoral CD8 ⁺ tumor-infiltrating lymphocytes were associated with increased OS and DFS.	(93)
Ma <i>et al</i> , 2019	PD-1(Hi) and CD8 ⁺ T cells	Retrospective	-	n=612	-	PD-1(Hi) or TIM3 ⁺ PD-1(Hi)CD8 ⁺ T cells were associated with poor prognosis, and the latter was positioned in close proximity to PD-L1 ⁺ tumor associated macrophages.	(96)
Ng <i>et al</i> , 2020	Intra-tumoral CD38 ⁺ cells and CD38 ⁺ CD68 ⁺ macrophage density	Retrospective	ICIs	n=49	PFS and OS	IHC and mIHC/IF analyses revealed that an increased intra-tumoral CD38 ⁺ cell proportion was strongly associated with an improved response to ICB. Patients with a high CD38 ⁺ CD68 ⁺ macrophage density had an increased mOS (by 24 months) compared with patients with a low CD38 ⁺ CD68 ⁺ macrophage density.	(97)
Gu <i>et al</i> , 2021	Five immune-associated genes (LDHA, PPAT, BFSP1, NR0B1 and PFKFB4)	Retrospective	ICIs	n=365	-	ROC and Kaplan-Meier analyses indicated that the model could stratify patients into a low-risk and a high-risk group, wherein the high-risk group exhibited a worse prognosis and was less sensitive to immunotherapy compared with the low-risk group.	(98)
Winograd <i>et al</i> , 2020	CTCs	Prospective	ICIs	n=10	Response rate	There was a strong association between the presence of PD-L1 ⁺ CTCs and a favorable treatment response in the subset of patients with HCC receiving immunotherapy.	(103)

HCC, hepatocellular carcinoma; PD-1, programmed death 1; PD-L1, programmed death-ligand 1; CTCs, circulating tumor cells; CTNNB1, catenin β 1; MSI, microsatellite instability; ICIs, immune checkpoint inhibitors; OS, overall survival; PFS, progression-free survival; TPS, tumor proportional score; TMB, tumor mutational burden; CR, complete response; DFS, disease-free survival; TIM3⁺, T cell immunoglobulin and mucin domain-containing protein 3; IHC, immunohistochemistry; mIHC/IF, multiplex IHC/immunofluorescence; mOS, median OS; ROC, receiver operating characteristic; ICB, immune checkpoint blocker.

of immunotherapy efficacy. A comprehensive treatment plan cannot be supported by a single biomarker (104). It is important to evaluate how different biomarkers interact, as is performed in the CRAFTY score, which combines CRP and AFP as aforementioned (105,106).

Analysis of the spatially distinct distribution of different immune cell types in the TME and the dynamic interactions between them has been demonstrated using multiplex IHC/immunofluorescence, which allows the simultaneous analysis of multiple immune parameters on the same paraffin-embedded tissue section (107). In HCC, Ng *et al* (97) revealed that the total CD38⁺ cell ratio and CD38⁺CD68⁺ macrophage density were indicators of responsiveness to immune checkpoint blockade, and were an improvement on the PD-L1 score or CD8⁺ T cell density. Additionally, the combined use of two markers can improve the prediction accuracy. In a recent study, the effects of TMB, gene expression profiling and PD-1, combined and alone, on the prognosis prediction in NSCLC were compared (108). It was revealed that the combination of at least two biomarkers was more accurate compared with the use of a single biomarker; however, combinations of three biomarkers were not revealed to be predictive (108). In patients with NSCLC, Hurkmans *et al* (109) investigated the interaction of PD-L1, CD8⁺ T cell infiltrates and human leukocyte antigens (HLA) class-I using IHC. The findings indicated that patients with an increased PFS had high tumor mutation loads, high infiltration of CD8⁺ T cells or no loss of HLA class-I (109). In addition to the combination of immune drugs, new anti-tumor therapies such as photodynamic therapy and photothermal therapy can increase the immune response of tumor cells by changing the TME, and demonstrate synergistic effects (110). Comprehensive ranking based on the fundamental molecular and cellular pharmacological foundations and relevant mechanisms of action to hit multiple targets, as well as further investigation of the next-generation immunotherapies for patients with primary and acquired drug resistance, may improve the prediction of the optimal strategies (111,112). Currently, there are no data available on the combined prediction of immunotherapy efficacy by several indicators in liver cancer. Table III provides a brief summary of tumor-associated biomarkers used in HCC immunotherapy.

5. Conclusion

In recent years, more immune-associated drugs, including atezolizumab in combination with bevacizumab, pembrolizumab and nivolumab, have been administered in clinical settings. While progress has been made in the treatment of liver cancer, not all patients respond effectively to immunotherapy. An important problem that needs to be solved is how to identify patients who would be sensitive to immunotherapy to avoid exposure to drug toxicity and a waste of medical resources. Non-invasive biomarkers are necessary. The collection and detection of NLR, PLR, ctDNA, CTC and intestinal microorganisms is less traumatic to patients, easier to collect and can achieve dynamic detection. PD-1/PD-L1, genetic characteristics and the TME provide more information on tumor heterogeneity. However, the treatment of liver cancer is a combination of multiple treatment methods and various treatment modes. In metastatic melanoma, Pires da Silva *et al* (113)

used conventional clinical parameters, factors such as the Eastern Cooperative Oncology Group Performance Status, presence/absence of liver and lung metastases, amongst others, to establish a model for predicting prognosis with validation in independent cohorts. The model successfully predicted the responses and survival rate outcomes of patients with metastatic melanoma after receiving immunotherapy (109,113,114). Furthermore, given the presence of tumor heterogeneity and the dynamic nature of the TME in liver cancer, as well as the complex interactions and regulation between the two, a single predictor is insufficient for the complexity of treatment methods. A combinatorial, precise and diverse strategy is thus necessary for immune biomarkers.

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Authors' contributions

MC conceived the topic for the present review and wrote the manuscript. JW and XZ were responsible for reviewing and editing the manuscript. ML revised the content of this review. All authors read and approved the final version of the manuscript. Data authentication is not applicable.

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Competing interests

The authors declare that they have no competing interests.

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