The immune landscape of hepatocellular carcinoma—where we are? (Review)

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Abstract. Hepatocellular carcinoma (HCC) is one of the most common types of cancer diagnosed worldwide. After a decade of stagnation, several novel compounds have recently been shown to be effective in the treatment of HCC. Since immunotherapy is associated with important clinical benefits in some, but not all patients, it is essential to identify reliable predictive biomarkers. As the complex interplay between hepatocytes and immune cells is highly dependent on the tumor microenvironment, the tumor microenvironment has been suggested to be an important factor associated with the response to therapy and is currently being extensively investigated. Within this network, several important factors should be highlighted. Most of the cells are hepatocytes, but fibroblasts, endothelial cells, and immune cells are also present. Tumor-infiltrating leukocytes include several populations of cells and each of them plays a role in forming the tumor environment. Some of these cells may have antitumor effects, whereas others may be associated with the progression of the disease. The most important subsets include tumor-associated macrophages, tumor-associated neutrophils, and lymphocytes. These groups are described in the present review. The immune response is controlled by immune checkpoint molecules. One of the most important molecules involved in this checkpoint process seems to be the programmed death-1 (PD-1) receptor, which typically is induced on activated T cells, natural killer (NK) cells, B cells, and antigen-presenting cells. On the other hand, programmed death ligand 1 (PD-L1) is expressed by tumor cells, hepatocytes and hepatic stellate cells, and Kupffer cells or liver sinusoidal cells. Complex interactions between ligands and receptors are dependent on the signals from the microenvironment leading to either cancer development or apoptosis. Evidence from several studies indicates that patients with higher expression levels of PD-L1 on tumor cells or immune cells are more likely to achieve beneficial results from treatment with checkpoint blockers. This review focuses on the basic information regarding the microenvironment and its components, particularly on immune system involvement.

Contents

1. Introduction
2. Myeloid-derived suppressor cells (MDSCs)
3. Tumor-associated macrophages (TAMs)
4. Tumor-infiltrating leukocytes (TILs)
5. Tumor-associated neutrophils (TANs)
6. Tumor microenvironment and response to systemic treatment
7. Immune checkpoint inhibition
8. Immune checkpoint inhibitors assessed for treatment of HCC
9. Immune microenvironment factors associated with the response to immunotherapy
10. Additional potential biomarkers and predictive factors for the response to immunotherapy
11. Conclusions

1. Introduction

Hepatocellular carcinoma (HCC) is one of the most common types of cancer diagnosed worldwide (1). For several years, the only treatment option for patients with advanced disease was sorafenib (2,3). However, more recently, promising results have been obtained from trials with immunotherapy. For example, the use of atezolizumab with bevacizumab provided better outcomes than sorafenib (4). Since immunotherapy may be associated with clinical benefits in certain patients, it is essential to identify reliable predictive biomarkers of treatment response. Therefore it is crucial to better understand the molecular and microenvironmental characteristics of the tumor.

Carcinogenesis is associated with cirrhosis, chronic inflammation, injuries, and regeneration of hepatocytes (5). The interplay between hepatocytes and immune cells depends on
the tumor microenvironment which forms the complex network involved in hepatocarcinogenesis. Within this network most of the cells are hepatocytes, but fibroblasts, endothelial cells and immune cells [such as tumor-associated macrophages, T lymphocytes] also play a critical role. The microenvironment also includes growth factors, enzymes, and extracellular matrix proteins, and cytokines may also contribute to carcinogenesis (6). Due to the association between the gut and liver, matrix proteins, and cytokines may also contribute to carcinogenesis (6). Due to the association between the gut and liver, matrix proteins, and cytokines may also contribute to carcinogenesis (6). Due to the association between the gut and liver, matrix proteins, and cytokines may also contribute to carcinogenesis (6). Due to the association between the gut and liver, matrix proteins, and cytokines may also contribute to carcinogenesis (6).

In this review, we have summarized the basic data regarding immune cells and their role in hepatocarcinogenesis. Tumor-infiltrating leukocytes (TILs) include several populations of cells with different roles. Some of these may have antitumor effects, while others may be associated with the progression of the disease. The most important subsets include tumor-associated macrophages (TAMs), tumor-associated neutrophils (TANs), and T lymphocytes, and are briefly described in Table I. Furthermore, we briefly focused on the prognostic and predictive factors associated with outcomes of immunotherapy.

2. Myeloid-derived suppressor cells (MDSCs)

MDSCs are a heterogeneous subset of myeloid cells that play an essential role in the immunosuppressive network. MDSCs may differentiate into granulocytes, dendritic cells, and macrophages. Due to the hypoxic microenvironment, the process of differentiation can be prevented in HCC (8,9). It was observed that in patients with HCC, MDSCs inhibit T-cell proliferation and induce T-regulatory cells (10). The proliferation of T cells is inhibited through the depletion of arginine due to increased arginase activity. Furthermore, the production of matrix metalloproteinase 9 (MMP-9) by MDSCs contributes to increased neangiogenesis through increasing vascular endothelial growth factor (VEGF) bioavailability (11). MDSCs can secrete IL-10, which results in M2 polarization as well as inhibition of natural killer (NK) cells, CD4+ and CD8+ lymphocytes through the expression of transforming growth factor β (TGF-β) (12,13). In a mouse model, targeting MDSCs was associated with a better response to sorafenib (14). A recent meta-analysis highlighted several observations. Firstly, the percentage of MDSCs in HCC patients was higher than in healthy individuals or patients with other chronic liver diseases. Moreover, higher quantities of MDSCs were inversely correlated with overall survival (OS) and relapse-free survival (RFS).

3. Tumor-associated macrophages (TAMs)

The population of liver macrophages includes Kupffer cells that reside within the liver and originate from the yolk sack and infiltrating hematopoietic stem cells/bone marrow-derived monocytes. TAMs are derived from monocytes recruited at the tumor site by molecules produced by neoplastic or stromal cells (15). However, they may be associated with a poorer prognosis, as some TAMs exhibit antitumor properties. There are two subsets of TAMs.

M1 macrophages are activated by cytokines such as interferon (IFN)-γ, tumor necrosis factor (TNF)-α, and granulocyte-macrophage colony-stimulating factor (GM-CSF), or in response to microbial infection. This mechanism is vital for pro-inflammatory and antitumor responses. Production of interleukin (IL)-12 and other pro-inflammatory cytokines leads to the initiation of a Th-1 dependent immune response. They are also capable of cytotoxic activity against cancer cells (16,17).

Conversely, M2 macrophages play an anti-inflammatory role and are associated with the promotion of tumor progression. They may be alternatively activated by IL-4, IL-10, IL-13, or macrophage colony-stimulating factor (M-CSF), and glucocorticoid hormones. M2-like macrophages are further sub-divided into four subtypes, with different roles (18,19). M2a macrophages are involved in tissue repair as well as cell growth and endocytic processes. M2b cells regulate the immune response. M2c cells are responsible for apoptosis. Finally, M2d macrophages enhance tumor growth and angiogenesis (20). TAMs may secrete pro-angiogenic cytokines such as VEGF, TGF-β, or platelet-derived growth factor (PDGF) which contributes to the progression of cancer (21). Promoting tumor progression by M2 macrophages is associated with increased recruitment of regulatory T cells leading to the suppression of T effector cells. This mechanism results from the secretion of cytokines such as IL-10, TGF-β, or chemokines such as C-C motif chemokine ligand (CCL)17, CCL8, CCL22, and CCL24 (22).

A high count of M2 cells is associated with enhanced angiogenesis and metastasis, which leads to a poorer prognosis. Moreover, the production of IL-17 by M2 TAMs has been shown to suppress oxaliplatin-induced apoptosis in HCC (23,24).

It was suggested that CD206+ M2, but not CD68+ TAMs may be used as a prognostic biomarker in HCC (25). A recently published study indicated that CD68+ M1 TAMs are associated with the induction of programmed death-ligand 1 (PD-L1) in HCC cells, which suggest they possessed a pro-tumor role (26). Another analysis showed that CD68+ TAMs were associated with a poor prognosis (27). These results highlight the need for further investigations.

There are several mechanisms involved in immune suppression associated with TAMs. One of these is the interaction with PD-L1, which was confirmed in vivo in mouse models where TAM-derived PD-L1 contributed more significantly to suppressing antitumor immunity than the host-derived PD-L1 (28). PD-L2 expressed by TAMs is also involved in suppressing the host antitumor response in the murine microenvironment (29).

It is suggested that TAMs are involved in resistance to sorafenib through sustaining tumor growth and metastasis by secreting hepatocyte growth factor (HGF). This in turn may activate the HGF/c-Met, ERK1/2/MAPK, and PI3K/AKT pathways in tumor cells. An interesting concept would be combining sorafenib with HGF inhibitors such as cabozantinib to improve treatment outcomes (30).

Most of the TAMs within the tumor microenvironment are M2 macrophages. Since M2 cells promote tumor development, downregulate M1 functions and adaptive immunity, and favor angiogenesis and regeneration of tissues, they could be a potential target for novel therapies. In a rat model, zoledronic acid treatment enhanced the effects of transarterial...
chemoembolization by inhibiting TAM infiltration and tumor angiogenesis (31).

As TAMs may influence tumor progression, several studies have attempted to neutralize their role. One of the approaches is to re-educate TAMs using the M-CSF receptor with pexidarstentinib (PLX3397), which inhibited tumor growth and increased the CD8+ lymphocyte count (32). In a preclinical setting, another agent that proved efficacious was baicalin (8-bromo-7-methoxychrysin) (33). Tocilizumab inhibits IL-6 produced by TAMs, which may contribute to the suppression of tumorigenesis (34). Inhibition of TAM recruitment was also suggested as a therapeutic option; this has been tested in studies targeting chemokine receptor type 2 (CCR2) antagonists and showed promising results (35,36).

4. Tumor-infiltrating leukocytes (TILs)

TILs may be involved in the antitumor responses through the direct mechanisms of the adaptive immune system and modulation of the innate response or angiogenesis (37). Various cell subsets play different roles. First, NK cells and cytotoxic T lymphocytes are able to target tumor cells and thus prevent their progression, especially in the early stages. Later, the antitumor response is less pronounced, which correlates with patient prognosis (38).

Another subset of cells, regulatory T lymphocytes (Tregs, CD4+), may promote immune tolerance and inhibit the anti-tumor-promoting roles of other cells. One of these mechanisms is the secretion of TGF-β and IL-10, which inhibits CD8+ T-cells (39). One subset, Th-17 cells, secretes IL-17 and this promotes disease progression by inducing angiogenesis, which is associated with poorer outcomes (36).

One of the roles of cytotoxic CD8+ lymphocytes is to prevent tumor progression by killing cancer cells. However, in the case of prolonged exposure to antigens, CD8+ lymphocytes differentiate into so-called exhausted CD8+ cells with impaired cytotoxic functions (40). These exhausted CD8+ cells may be associated with decreased expression of cytokines, but also with increased expression of inhibitory receptors, including programmed cell death 1 (PD-1), lymphocyte-activation

| Cell type               | Impact                                                                 | Potential prognostic/predictive factor | (Refs.) |
|-------------------------|------------------------------------------------------------------------|----------------------------------------|---------|
| MDSCs                   | Negative impact on immunoregulation                                     | Yes                                    | (10-14) |
|                         | Increased neoangiogenesis                                              |                                        |         |
|                         | M2 polarization                                                        |                                        |         |
|                         | Targeting MDSCs are associated with increased response to sorafenib    |                                        |         |
|                         | Higher MDSC levels are inversely correlated with overall and relapse-free survival |                                        |         |
| TAMs                    | Antitumor response, but CD68+ M1 polarization                          | Yes                                    | (16,17,26) |
| M1 macrophages (pro-inflammatory) | TAMs may be associated with the induction of PD-L1 in HCC cells (pro-tumor role) |                                        |         |
| M2 macrophages (anti-inflammatory) | Promotion of tumor progression                                         | Yes                                    | (18,19,23,24,30) |
|                         | Enhanced angiogenesis and metastasis                                   |                                        |         |
|                         | Poorer prognosis                                                       |                                        |         |
|                         | Resistance to sorafenib                                                |                                        |         |
| TILs                    | Targets tumor cells to prevent tumor progression                        | Yes                                    | (38)    |
| NK cells                | Cytotoxic T lymphocytes (CD8+)                                          |                                        |         |
|                         | May promote immune tolerance and inhibit the anti-tumor-promoting roles of other cells |                                        |         |
|                         | Promotes disease progression by promoting angiogenesis                  |                                        |         |
| Regulatory T lymphocytes (CD4+) | Able to promote or inhibit the progression of the disease               | Yes                                    | (51,52) |
|                         | Resistance to sorafenib                                                |                                        |         |

HCC, hepatocellular carcinoma; MDSCs, myeloid-derived suppressor cells; TAMs, tumor-associated macrophages; PD-L1, programmed death ligand 1; TILs, tumor-infiltrating lymphocytes; NK, natural killer; TANs, tumor-associated neutrophils.
gene 3, cytotoxic T lymphocyte-associated antigen (CTLA-4), and T cell immunoglobulin domain (41,42). TILs with high levels of PD-1 expression exhibit higher expression levels of genes regulating T-cell exhaustion (43).

The low presence of intratumoral Tregs with high intratumoral activated CD8+ cytotoxic cells (CTILs) was found to be associated with improved disease-free survival (DFS) and OS (44). This is supported by the observation, that increased levels of regulatory T cells were correlated with CD8+ T-cell impairment and poor survival in HCC patients (45). Low CD8+ cell counts were found to be associated with poorer survival in another study (46). It was also observed, that CD8+ infiltration was associated with a so-called ‘immune cell stroma’ HCC type, which in comparison with ‘conventional stroma’, is characterized by the lack of catenin β1 (CTNNB1) mutations, global hypermethylation, expression of PD-1 and PD-L1 in TILs, and expression of PD-L1 in tumors. This type was also associated with an improved prognosis (47). The results of several studies indicate that the inhibition of Treg-induced suppression may be effective in restoring anti-tumor immune responses in several types of cancer (48-50).

5. Tumor-associated neutrophils (TANs)

The role of TANs in carcinogenesis is complex. TANs can promote as well as inhibit the progression of the disease, dependent on the cytokines released. The secretion of CCL-2 or CCL17 is associated with poorer outcomes. Those cytokines may also promote the infiltration of Tregs and macrophages. This infiltration negatively correlates with survival. It is suggested that TANs are involved in sorafenib resistance (49). In vitro, when HCC cell lines were cocultured with TANs, colony formation, cell migration, invasion, and sphere formation were enhanced, while apoptosis was inhibited.

There is growing evidence, that miRNAs may regulate tumor progression in HCC. For example, miR-301b-3p was correlated with the tumor size and advanced stages of the disease. Knockdown of miR-301b-3p reduced proliferation, induced cell cycle arrest in the G2/M phase, and induced apoptosis. Furthermore, TANs secrete bone morphogenetic protein 2 and TGF-β2 and increased miR-301-3p expression in HCC cells, which subsequently suppressed gene expression of limbic system-associated membrane protein (LSAMP) and CYLD lysine 63 deubiquitinase (CYLD), and increased the acquisition of stem cell characteristics in HCC cells. TAN-induced HCC stem-like cells were hyperactive and further increased TAN infiltration, suggesting a positive feedback loop. In clinical HCC samples, increased quantities of TANs were correlated with elevated miR-301b-3p levels, decreased LSAMP and CYLD expression, and increased nuclear p65 accumulation and C-X-C motif chemokine ligand 5 (CXCL5) expression, all of which were associated with patient outcomes (50).

6. Tumor microenvironment and the response to systemic treatment

Sorafenib has been the standard of care for advanced HCC for several years. However, there are several challenges associated with sorafenib treatment, including low response rates, toxicity, and acquisition of drug resistance. The tumor microenvironment plays an essential role in mechanisms associated with resistance. Several studies have demonstrated that the infiltration of TANs and TAMs may be correlated with the sensitivity to sorafenib (51). Furthermore, treatment with sorafenib results in hypoxia related to the depletion of pericytes and a decreased number of vessels. It is suggested that sorafenib-induced hypoxia may be associated with subsequent exosome-mediated resistance to sorafenib (52). Characterization of the tumor microenvironment has led to the division of HCC into four immune subclasses. About 20% of patients present with an immunogenic subclass characterized by massive T cell infiltration and activation of the immune checkpoint pathway. These patients exhibit the best response not only to immunotherapy, but also to sorafenib (53).

In 2018, the results of a phase III trial with lenvatinib showed it to be effective in the first-line treatment of HCC. It was also evaluated in combination with immunotherapy. Lenvatinib reduced the number of TANs and increased the percentage of activated CD8+ T cells secreting IFN-γ and granzyme B. It is worth noting that the exhaustion of CD8+ lymphocytes is one of the mechanisms involved in immune escape and cancer development. On the other hand, it is regulated by the PD-1 signaling (54). These findings provide a scientific rationale for the combination therapy of lenvatinib with PD-1 blockade (55). In a mouse model of HCC, the immunomodulatory activity of lenvatinib led to an enhanced response to anti-PD-1 treatment. In 2021, it was also suggested in a small retrospective study in which patients with HCC received lenvatinib with pembrolizumab or nivolumab (56,57).

7. Immune checkpoint inhibition

Immune checkpoint molecules control the immune response. One of the most important seems to be the PD-1 receptor, which typically is induced on activated T cells, NK cells, B cells, and antigen-presenting cells. Conversely, PD-L1 is expressed by tumor cells, hepatocytes, hepatic stellate cells, and Kupffer cells or liver sinusoidal cells (58). PD-L2 is another known ligand for PD-1, and it is present on dendritic cells. Complex interactions between ligands and receptors dependent on the signals from the microenvironment lead to either cancer development or apoptosis.

Evidence from several studies indicates that patients with higher expression of PD-L1 on tumor cells or immune cells are more likely to achieve benefits from treatment with checkpoint blockade (59,60).

The impact of PD-L1/PD-L1 on the prognosis and treatment outcomes in HCC has been studied in several studies. However, the results have proven to be conflicting, and the underlying mechanism is not fully understood. It has been shown that patients with high expression of PD-L1 and a high TIL presence tend to have better prognoses, whereas low expression of PD-L1 and galectin-9 and low CD8+ TIL counts are associated with poor HCC-specific survival (46). On the other hand, the results of another study, where the PD-L1 expression was correlated with clinical and pathological features suggested that PD-L1 expression by either neoplastic
or intratumoral inflammatory cells was associated with tumor aggressiveness (61). These conflicting results clearly show the need for further investigation of the immune landscape of the HCC microenvironment.

Of note, it has been suggested that stratifying tumors according to the expression of PD-L1 and TILs could be helpful for predicting the response to treatment. Type 1 cancers are characterized by PD-L1+TILs+ and benefit from the single-agent anti-PD-1/L1 blockade. Type 2 cancers (PD-L1+TIL-) have been predicted to have poor responses to single-agent immunotherapy and poorer prognoses. In type 3 cancers (PD-L1+TIL+), PD-L1 positivity is not a single predictive factor for determining the response to anti-PD-1/PD-L1 treatment, as without TILs, a reaction to blocking PD1/L1 is unlikely. Finally, other suppressive mechanisms may be dominant for type 4 cancers (PD-L1+TIL-) (62). Thus, a simple distinction between the presence or absence of TILs or PD-L1 may not be sufficient. It is well established that other immune checkpoint molecules may be co-expressed on T cells. For example, lymphocyte activation gene-3 (LAG-3), a negative regulator of T cells is also expressed on T cells and its inhibition increases the antitumor response (63). It has been shown that HCCs may contain CD8+ T cells that express different levels of PD-1. Those cells with a discrete population of PD-1 high CD8+ T cells express T cell immunoglobulin mucin-domain-containing protein-3 (TIM-3) or LAG-3 and can produce low levels of IFN-γ or TNF in response to anti-CD3. Incubation of these cells with antibodies against PD1 and TIM-3 or LAG-3 further restored proliferation and production of cytokines. The results of this study indicate that HCCs with a discrete population of PD1-high CD8+ T cells may be more susceptible to combined immune checkpoint blockade-based therapies (43).

Kurebayashi et al suggested that the tumor microenvironment can be classified into three subtypes based on immunohistochemical analyses of the immune regulatory molecules; namely, Immune-high, Immune-mid, and Immune-low. The Immune-high subtype is characterized by increased B-/plasma-cell and T-cell infiltration. The Immune-high subtype and B-cell infiltration were identified as independent B-/plasma-cell and T-cell infiltration. The Immune-high subtype had significantly better prognoses. Finally, patients with high-grade HCC of the predominant high-grade HCC, and Hoshida's S1/Boyault's G2 subclasses. The Immune-high subtype had significantly better prognoses. Finally, patients with high-grade HCC of the predominant high-grade HCC, and Hoshida's S1/Boyault's G2 subclasses. Depending on the Treg/CD4+ ratio, the Immune-low type was subdivided into 2 classes (1-lower Treg/CD4+ ratio; 2-higher) (64).

As mentioned above, TIM-3 is another regulatory molecule that plays a role in tumor progression. Its levels may be increased in CD4+ and CD8+ cells or TAMs, and this is correlated with a poorer prognosis. On the other hand, its suppression resulted in an increased antitumor response (13).

8. Immune checkpoint inhibitors assessed for treatment of HCC

Recently several immune checkpoint inhibitors have been evaluated as treatments for HCC. Most of the studies have focused on PD-1/PD-L1 inhibitors and CTLA-4 inhibitors. Anti-PD-1 treatment with monoclonal antibodies was found to result in the blockade of PD-1 interaction with PD-L1 or PD-L2 expressed in antigen-presenting cells, which is involved in the T-cell antitumor response. Checkpoint inhibition enhances T-cell response and normalizes the immune response within the microenvironment (65).

From a clinical point of view, the most important inhibitors assessed were atezolizumab (anti-PD-L1) and bevacizumab (anti-VEGF) in the ImBrave150 trial, which showed a significant response to the combined treatment, and has since become the recommended standard of care (66,67). Bevacizumab was shown to reduce tumor microvessel density and modulate the immune microenvironment through the reduction of infiltration of tumor-associated macrophages and the increase in tumor-associated neutrophils (68). VEGF-A produced in the tumor microenvironment enhances the expression of PD-1 and other inhibitory checkpoints involved in CD8+ T-cell exhaustion, and this could be inhibited using anti-angiogenic agents targeting VEGF-A and/or VEGFR (69). Thus, anti-VEGF therapy leads to the reduction of immunosuppressive factors within the tumor and its microenvironment and is associated with increased T-cell infiltration, which together enhances the response to immunotherapy. Atezolizumab is a monoclonal antibody that directly binds to PD-L1 and provides a dual blockade of the PD-1 and B7.1 receptors, releasing PD-L1/PD-L1-mediated inhibition of the immune response, including the reactivation of the antitumor immune response without inducing antibody-dependent cellular cytotoxicity. The effect of anti-VEGF and anti-PD-1/PD-L1 treatment is illustrated in Fig. 1.

Of note, combined treatment with bevacizumab and atezolizumab was associated not only with improved survival [median progression-free survival (PFS) was 6.8 months vs. 4.3 months in the sorafenib group; hazard ratio (HR) 0.59; 95% confidence interval (CI), 0.47-0.76; P<0.001], but also with the maintenance of the quality of life (11.2 months vs. 3.6 months in patients treated with sorafenib; HR 0.63; 95% CI, 0.46-0.85) (70).

The Check-Mate 459 trial compared nivolumab (anti-PD-1) with sorafenib as a first-line treatment for HCC. Although the objective response rate was higher in the investigational arm, the increase in OS was not significant (71). Nivolumab was also evaluated in combination with ipilimumab (anti-CTLA-4) in the phase 1/2 trial CheckMate 040, where manageable safety, promising objective response rates, and durable responses were shown (72).

Pembrolizumab is a humanized monoclonal antibody that blocks the interaction of the PD-1 receptor with PD-L1 and PD-L2, which results in the enhancement of the antitumor effect of the immune system associated with T-cell responses (73). Based on the promising results of the phase II trial Keynote 224 (74), pembrolizumab was evaluated as a second-line treatment for HCC in the Keynote 240 trial. However, the results did not show a notable clinical benefit (75).

Combined treatment with tremelimumab, an anti-CTLA4 antibody, and durvalumab showed promising results in patients...
with advanced HCC in a phase I/II trial (76). It is also being investigated in the phase III HIMALAYA trial (77).

Several other immunotherapeutic agents are under evaluation in various combinations with tyrosine kinase inhibitors or other immunotherapeutic drugs. Select phase III trials are summarized in Table II (78-91). The results of the already published trials suggest that there are groups of patients that may benefit more from immunotherapy compared with others. Currently, it seems to be crucial to identify predictive factors for the response to targeted therapy. It is suggested that the microenvironment may play an important role in the response to the treatment.

A very interesting topic is the use of immunotherapy in patients that qualify for liver transplants. There are limited data regarding the use of immunotherapy for downstaging or bridging therapy from clinical case reports (92,93). The use of adoptive immunotherapy with liver allograft-derived NK cells was evaluated in a study presented during the 2015 American Transplant Congress, and it was suggested that this approach may improve RFS (94). Immunotherapy was also used for recurrent HCC treatment after liver transplantation. Interestingly, a case report where ipilimumab was used in a patient with recurrent HCC after liver transplantation described a durable response and no rejection of the organ (95). However, it should be noted that immunotherapy may increase the risk of liver rejection and therefore should be considered rather as a salvage therapy rather than a first-line approach (96). Clearly, there is a need for additional data.

Figure 1. Anti-VEGF and anti-PD-1/PD-L1 treatment: Mechanism of action. PD, programmed death; PD-L1, programmed death-ligand 1; VEGF, vascular endothelial growth factor; VEGFR2, vascular endothelial growth factor receptor 2.
9. Immune microenvironment factors associated with the response to immunotherapy

The response to treatments depends on several mechanisms and the specific characteristics of the tumor, the local microenvironment, as well as the host itself. Firstly, since most of the immunotherapeutics that showed clinical efficacy are checkpoint inhibitors, it has been suggested that the expression of PD-1 may be a reliable predictive factor. It has been shown that in patients with head and neck cancers (97), melanoma (98), or lung cancer (99), increased PD-1 expression was associated with better treatment outcomes.

Interestingly, in the Check-Mate 040 trial, expression of PD-L1 in >1% of the tumor cells was detected in 20% of patients. However, the objective response between patients with higher expression of PD-L1 in comparison to those with low expression did not differ significantly: 26 vs. 19% (95% CI 13-26). This suggests that other mechanisms may be involved in the response to immunotherapy (66). Potentially, this could be related to the expression of PD-1 and PD-L1 on tumor-infiltrating lymphocytes.

Furthermore, in a small phase II trial where patients with advanced HCC were treated with pembrolizumab after sorafenib failure, the response was not correlated with either PD-L1 tumor staining, prior sorafenib therapy or a history of hepatitis. Correlative studies revealed high baseline plasma TGF-β levels (≥200 pg/ml) were significantly correlated with poor treatment outcomes after pembrolizumab. Tumor PD-L1 and plasma PD-L1/PD-1 levels were associated with plasma IFN-γ or IL-10. However, those results need caution due to the low number of patients (n=28) (100).

Recently it was demonstrated that androgen receptors may inhibit the expression of PD-L1 in HCC. It was found that the androgen receptor is overexpressed in the nucleus of ~37% of HCC tumors, which is significantly associated with an advanced disease stage and poorer survival rates. It has also been suggested that the overexpression of androgen receptors may be related to a worse response to PD-L1 inhibitors (101).

Studies conducted in non-small cell lung cancer highlighted the role of EGFR signaling in the regulation of the host immunity and tumor microenvironment. Cell lines with mutant EGFR exhibit increased expression of PD-L1 compared to wild-type EGFR cells (102).

There are several questions regarding the potential use of PD1/PD-L1 expression as a predictive factor. Firstly, the expression of PD-L1 in a CIK cell agent (Immuncell-LC) vs. non-treatment group in HCC (86). Clinicaltrials.gov number Design Status (Refs.)

| Clinicaltrials.gov number | Design | Status (Refs.) |
|--------------------------|--------|----------------|
| NCT02851784              | Combination therapy of microwave ablation and expanding activated autologous lymphocytes | Completed (78) |
| NCT02562755              | Vaccinia virus-based immunotherapy (Pexa-Vec) followed by sorafenib vs. sorafenib | Completed (79) |
| NCT05033522              | Living allogeneic Th1-like cells with anti-CD3/CD28 microbeads attached derived from precursors purified from healthy blood donors that are differentiated and expanded ex-vivo vs. FOLFOX regimen | Not yet recruiting (80) |
| NCT02576509              | Nivolumab vs. sorafenib as first-line treatment | Active, not recruiting (81) |
| NCT02678013              | RFA+highly-purified CTL vs. RFA alone for recurrent HCC | Active, not recruiting (82) |
| NCT04167293              | Combination of sintilimab and stereotactic body radiotherapy | Recruiting (83) |
| NCT03949231              | Infusion of PD-1/PDL-1 inhibitor via hepatic arterial vs. vein | Recruiting (84) |
| NCT04229355              | DEB-TACE plus lenvatinib or sorafenib or PD-1 inhibitor for unresectable HCC | Recruiting (85) |
| NCT00699816              | Adjuvant adoptive immune therapy using a CIK cell agent (Immuncell-LC) vs. non-treatment group in HCC | Completed (86) |
| NCT02709070              | Resection+highly purified cytotoxic T lymphocytes vs. resection alone | Active (87) |
| NCT04268888              | Nivolumab in combination with TACE/TAE | Recruiting (88) |
| NCT03867084              | Adjuvant pembrolizumab vs. placebo in HCC with complete radiological response after surgical resection or local ablation | Recruiting (89) |
| NCT04682210              | Adjuvant sintilimab plus bevacizumab in HCC after resection | Not yet recruiting (90) |
| NCT01749865              | Adjuvant cytokine-induced killer cell treatment in HCC after resection | Completed (91) |

HCC, hepatocellular carcinoma; RFA, radiofrequency ablation; CTL, cytotoxic T lymphocytes; HCC, hepatocellular carcinoma; PD, programmed death; L, ligand; CIK, cytokine-induced killer cell; DEB-TACE, drug-eluting bead transarterial chemoembolization; TAE, trans-arterial embolization.
in circulating T cells in patients who did not respond to PD-1/PD-L1 pathway blockade (103). Furthermore, currently, there is no standard for assessing the cut-off value for PD-L1 positivity and there are various kits used, which makes the comparison between studies more difficult. It was also shown that the expression of PD-L1 may change during the disease (104,105). On the other hand, it has been suggested that other factors associated with the tumor microenvironment may be necessary for predicting the response to immunotherapy. Recently, a classification of tumor microenvironments based on TILs and PD-L1 expression was described. Furthermore, it is suggested, that there is an immune class of HCC tumors which may be further divided into two subtypes, characterized by markers of an adaptive T-cell response or exhausted immune response. The exhausted immune response subclass expresses several genes regulated by TGF-β1 that mediate immunosuppression. According to the authors, these findings indicate that some HCCs may be susceptible to therapeutic agents designed to block the regulatory pathways in T cells, such as PD-L1, PD-1, or TGF-β1 inhibitors (106). In another study, a high number of PD-1+ TILs was shown to be associated with prolonged OS and DFS in patients with HCC who received surgery and adjuvant cytokine-induced killer cells treatment (107).

Of note, it was suggested that the response to immunotherapy may depend on the specific liver disease background, such as a viral infection. However, a recently published meta-analysis of 8 studies suggested that there was no significant difference in objective response rate between virally infected HCC and non-viral HCC patients [OR=1.03 (95% CI, 0.77-1.37; I²=30.9%, pH=0.152)]. Similarly, there was no difference between HBV-HCC and HCV-HCC patients in terms of objective response rate [OR=0.74 (95% CI, 0.52-1.06; I²=7.4%, pH=0.374)]. The infiltration of immune cells in the tumor microenvironment did not differ by etiology except for M0 macrophages, M2 macrophages, regulatory T cells, naive B cells, follicular helper T cells, activated dendritic cells, activated mast cells, and plasma cells. Despite differences in infiltration observed in specific cell types, the immune score and stromal score were generally comparable among the different etiological groups (108).

Interestingly, it was suggested that immune checkpoint inhibitors may be ineffective in non-alcoholic steatohepatitis (NASH)-related HCC. This was observed in a preclinical model as well as in a meta-analysis of three randomized phase III clinical trials that tested inhibitors of PD-L1 or PD-1 in >1,600 patients with advanced HCC. According to the results, immunotherapy did not improve survival in patients with non-viral HCC. In two additional cohorts, patients with NASH-driven HCC who received anti-PD-1 or anti-PD-L1 treatment showed reduced OS compared with patients with other aetiologies (109).

10. Additional potential biomarkers and predictive factors for the response to immunotherapy

Since radical treatment options are limited, and the prognosis for patients with advanced HCC remains poor, there is a need to identify reliable biomarkers of HCC to better tailor patient therapy. Several potential biomarkers for the response to immunotherapy have been investigated in HCC. Several other mechanisms may be involved in the response to immunotherapy. The gut-liver axis and the colon microbiome are some of the suggested factors associated with the response to immunotherapy. In a small study, where 8 patients with progressive disease after sorafenib failure were treated with nivolumab, fecal samples were collected and analyzed at baseline, after 1 week, and every 3 weeks after that. Variations in the gut bacteria were analyzed by metagenomic sequencing. It was observed that fecal samples from patients responding to immunotherapy showed higher taxa richness and increased gene counts than non-responders (110). Another mechanism potentially involved in the response to immunotherapy includes exosomes due to their role in the communication between host and tumor cells. Exosomes are involved in the transfer of proteins, DNA, and RNA. Exosomes could serve as biomarkers for early-stage HCC and as a possible target for therapy (111). There are several exosomal biomarkers for the prediction of survival in HCC, i.e. proteins involved in angiogenesis (SI00A11, SI00 calcium-binding protein A11) and numerous microRNAs such as miR-29b-3p, miR-30d-5p which are involved in cell migration, or miR-210 which is associated with angiogenesis (112,113). Another example may be exosomal circulating RNA (circPTGRI) which was suggested to promote HCC metastasis. Finally, exosome-based strategies to deliver drugs into tumors and the microenvironment showed promising results in preclinical and clinical trials (114-116). The complexity of the interplay between host and tumor requires further studies to identify reliable predictive factors. Other factors include tumor mutational burden (TMB), defined as the total number of mutations in the tumor exome. However, it seems to be relatively rare in HCC; in a recently published report median TMB was 4 mutations/Mb and only 6 tumors (0.8%) were classed as TMB-high. Out of 542 cases assessed for microsatellite instability (MSI), one (0.2%) was MSI-high and TMB-high. TMB may be associated with MSI or DNA mismatch repair gene deficiency. In a recently published analysis, the most commonly altered genes were TERT (44%), TP53 (35%), CTNNB1 (31%), ARID1A (12%), and MYC (12%) (117).

Currently, there are a vast group of biomarkers being investigated for HCC. They include post-translational modifications such as phosphorylation, glycosylation, ubiquitination, or acetylation. These changes are involved in various physiological processes, but also in disease progression (118). Next, generation sequencing techniques (NGS) are proving to be very powerful and valuable methods. Potentially, thanks to NGS profiling, patient responses to treatments may be predictable. An analysis showed that in patients with HCC, the WNT/β-catenin pathway (45%) and TP53 (33%) alterations were frequent and represented mutually exclusive molecular subsets. In sorafenib-treated patients (n=81), oncogenic PI3K-mTOR pathway alterations were associated with lower disease control rates (DCR, 8.3 vs. 40.2%), shorter median PFS (1.9 vs. 5.3 months), and shorter median OS (10.4 vs. 17.9 months). Conversely, patients treated with immune checkpoint inhibitors (n=31) activating altered WNT/β-catenin signaling had lower DCR (0% vs. 53%), shorter median PFS (2.0 vs. 7.4 months), and shorter median OS (9.1 vs.
Potential factors involved in response to immunotherapy. mi-RNA, microRNA; NASH, non-alcoholic steatohepatitis; PD-1/PD-L1, programmed death 1/programmed death ligand 1; TMB, tumor mutational burden.

15.2 months) (119,120). Potential factors involved in response to immunotherapy are summarized in Fig. 2.

Since histological specimens may not always be available in HCC, factors that could be assessed from blood examinations would be of value. A number of soluble factors such as cytokines are of interest. In a phase II study, where several circulating biomarkers were evaluated, low baseline levels of TGF-β were significantly associated with improved OS and PFS after treatment with pembrolizumab in advanced, unresectable HCC (100,120). However, a detailed analysis is beyond the scope of this review. A recently published analysis showed that in patients with unresectable HCC, treated with nivolumab or pembrolizumab, an early decrease in α-fetoprotein levels was associated with an increased objective response and increased survival. Moreover, albumin/bilirubin grade and Child-Pugh classification determined survival based on immunotherapy treatment (121).

11. Conclusions

This review briefly summarizes the current body of knowledge regarding immune cells within the tumor microenvironment in HCC. However, other factors may play important roles, such as tumor mutational burden or microsatellite instability (122). The changing landscape of the treatment possibilities in advanced HCC makes the role of predictive factors rise. Thus, there is a need to identify reliable factors that may help tailor treatments to the specific disease characteristics presented.

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Authors’ contributions

MG was responsible for conception and the design, data analysis and interpretation of the data as well as draft preparation of this review. KW conducted the draft preparation, critical revisions and approved the final manuscript version to be published; and agrees to be accountable for all aspects of the work. LK was responsible for draft preparing, critical revisions; approved of the final manuscript version to be published; and agrees to be accountable for all aspects of the work. LR conducted the draft preparing, critical revisions; approved of the final manuscript version to be published; and agrees to be accountable for all aspects of the work. RS conducted the draft preparing, critical revisions; approved of the final manuscript version to be published; and agrees to be accountable for all aspects of the work. All authors have read and approved the final manuscript. Data authentication is not applicable.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

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Competing interests

The authors declare that they have no competing interests.

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