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Review

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Time for hepatocellular carcinoma immunotherapy: insights for successful clinical applications in this challenging tumor

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Abstract

The multiplicity and phenotype of intratumoral immune infiltrate have been shown to influence the clinical outcome of hepatocellular carcinoma (HCC), thus providing a strong rationale to therapeutic interventions aimed at restoring the dysfunctional immune response against the tumor. Improving the knowledge of the complex interactions between transformed hepatocytes, nonparenchymal resident cells, and infiltrating immune cells (characterizing the HCC microenvironment) will be instrumental to increase the success rate of existing immunotherapeutic strategies and to identify new potential targets for intervention or biomarkers to optimize the selection of candidate patients.

Keywords: Hepatocellular carcinoma, immune checkpoint inhibitors, T lymphocytes, cytotoxic T lymphocytes, natural killer cells, macrophages, cytokines

INTRODUCTION

The liver immune landscape fosters tolerance towards foreign antigens driven by portal blood. Liver sinusoidal endothelial cells (LSECs) that separate liver parenchyma from sinusoidal blood, liver resident macrophages (Kupffer cells), hepatic stellate cells (HSCs), and dendritic cells (DCs) exert antigen presenting cell (APC) function and participate in the tolerogenic liver environment[1]. Innate immune cells such as natural killer (NK), NKT and γ/δ T cells are found at higher frequency in the liver, as Foxp3+ regulatory T cells (Tregs). The liver environment is also characterized by increased expression of immunosuppressive cytokines such as interleukin (IL)-10 and transforming growth factor (TGF)-β[2].

Hepatocellular carcinoma (HCC) initiation and progression are multi-step processes profoundly influenced by the interplay between hepatocytes and immune cells. Immunotolerance is disrupted in chronic liver disease where persistent infections with hepatitis B virus (HBV) or hepatitis C virus (HCV), accumulation of fat, exogenous toxic substances (alcohol) or iron overload (haemochromatosis) enhance inflammatory signals triggering a cycle of cell death/regeneration and compensatory fibrosis, leading to liver cirrhosis, that represents a pre-neoplastic state. Chronic inflammation induces the accumulation of reactive oxygen species, generating epigenetic changes and chromosomal instability that contribute to tumor initiation, with expression of neo-antigens and/or deregulation of the expression of oncofetal and cancer testis antigens such as alpha-fetoprotein (AFP), glypican-3 (GPC-3), melanoma-antigen gene (MAGE) family, NY-ESO.1[3].

IMMUNE RESPONSE AGAINST HCC

Cytotoxic T cells (CTLs) recognizing tumor-associated antigen (TAAs) have been detected in HCC patients and their abundance is associated with patient survival[3-5]. CD8+ and CD4+ T cells were shown to accumulate in early HCC with a progressive decrease in late stages, that represents a negative predictor for disease outcome[3,6]. TAA-specific CD8+ T cells from peripheral blood produce interferon (IFN)-γ upon stimulation, but tumour-infiltrating lymphocytes fail to do so, indicating the progressive exhaustion of intratumour CD8+ T cells[3]. Exhausted T cells are characterized by impaired effector function and sustained expression of co-inhibitory receptors, and cannot mature into memory T cells.

NK cells account for 25%-50% of the total number of liver lymphocytes and are strongly implicated in the anti-tumor response. Impaired effector function of NK cells was reported in HCC and related to disease outcome[7,8]. Several mechanisms have been implicated in NK cell dysfunction: the genetic make-up of KIR NK cell receptors[9-11], a higher percentage of NK-cells co-expressing inhibitory NKG2A and activating NKp30-NKp46 receptors[12,13], myeloid-derived suppressor cells (MDSCs)-mediated suppression[14-16], increased prevalence of a dysfunctional CD11bnegCD27neg NK-cell subset[17].

Among factors contributing to the immune suppressive microenvironment of HCC are cell-mediated mechanisms, the secretion of cytokines and chemokines by tumor, stromal, and infiltrating cells, and the immunoediting of TAAs[18,19]. In this context, adaptive immune response exerts a dual role with seemingly opposite functions, being part of the inflammatory environment that likely plays a major role in tumor promotion, but hampering tumor dissemination through cytotoxic function against transformed cells[20].

MECHANISMS OF IMMUNE IMPAIRMENT IN HCC

The development and progression of HCC evolve through a dynamic interaction between tumor cells, non-parenchymal resident cells such as Kupffer cells (KCs), HSCs, LSECs, infiltrating immune cells and immune mediators. All these elements participate in the tumor microenvironment that exerts a profound influence on the evolution of disease. The many factors that co-operate to the immune landscape of HCC represent potential targets for therapeutic intervention.

Immunosuppressive molecules

Immune checkpoints are coinhibitory molecules that control the duration and the strength of immune response to prevent over-activation of T cells. This class of molecules includes CTLA-4, PD-1, TIM-3, lymphocyte activation gene 3 protein (LAG-3) and B and T lymphocyte attenuator (BTLA). Immune checkpoints are exploited by tumors as mechanisms of immune evasion and may therefore become major targets of immune therapeutic strategies.

CTLA-4 is expressed by activated T cells and by Treg cells. It competes with the activating molecule CD28 for binding CD80 and CD86[21] and activates Tregs[22,23].

PD-1 is expressed by activated T and B lymphocytes, NK cells, Treg cells, MDSCs, monocytes and DCs[24,25]. The expression of PD-1 is induced by several cytokines including IFN-γ[26,27]. Under hypoxic conditions, production of hypoxia-inducible factor (HIF)-1 induces the expression of PD-L1, the PD-1 ligand, in MDSCs and tumor cells[22,27,28]. The interaction between PD-1 and PD-L1 inhibits T cell effector function and leads to T cell exhaustion[29]. The immune infiltrate of HCC is enriched in PD1+ CD8+ cells and their abundance is associated with disease prognosis[30]. The expression of PD-L1 in HCC has also been reported as a prognostic factor of shorter disease-free and overall survival[31-34].

TIM-3 is a transmembrane protein expressed on various immune cells and interacting with multiple ligands among which galectin-9, a soluble protein expressed by several tissues including liver[35] that negatively regulates Th1 cell function[36]. In addition, TIM-3+ Treg cells exhibit enhanced suppressor activity[37]. A role of the TIM-3/galectin-9 pathway in the determination of HCC-infiltrating T cell dysfunction has been reported[38].

LAG-3 binds MHC class II molecules with high affinity[25,39] thus reducing co-stimulatory function of DCs. LAG-3 is upregulated upon activation of T cells[40] and is a marker of exhausted T cells[41]. Its activation and, as a consequence, its blockade are synergistic with PD-1[42,43]. BTLA is upregulated on activated lymphocytes and on tumour-specific CD8+ T cells in patients with cancer[44]. High expression of the BTLA ligand HVEM (herpesvirus entry mediator) has been reported in patients with HCC and is associated with reduced lymphocyte infiltration and poorer prognosis[45].

Cytokines are membrane-bound or secreted proteins involved in the regulation of immune cell function, inflammation and angiogenesis. Their pleiotropic roles include pro- and anti-inflammatory functions. CD4+ T helper cells produce either Th1 cytokines [e.g., interleukin (IL)-1, IL-2, IL-12, IL-15, tumor necrosis factor (TNF)-α and IFN-γ] usually defined pro-inflammatory, or Th2 cytokines (e.g., IL-4, IL-8, IL-10 and IL-5) mainly exerting anti-inflammatory functions[46].

Increased levels of IL-10 and TGF-β and reduced levels of IFN-γ have been detected in plasma from HCC patients[47]. In liver tissue IL-10 is produced by DCs, KCs, HSCs, LSECs, MDSCs and T cells, inducing tolerance[48,49]. Tolerogenic effect of IL-10 is linked to inhibition of CD4+ T cell activation[50] and, as a consequence, of cytotoxic CD8+ T-cell function[51]. In addition IL-10 further interferes with T cell activation by downregulating the expression of MHC-II and CD80/CD86 on APCs[52] and of NF-kB[53], a transcription factor strongly implicated in inflammatory responses. Despite its immunosuppressive activity in the context of inflammation, several studies report an immune-stimulatory role of IL-10 on CD8+ T-cell and NK-cell cytotoxic activity in experimental tumor models[54-56].

TGF-β, produced by parenchymal and non-parenchymal liver cells, is implicated in the maintenance of liver immune homeostasis[57] and may exert a suppressive function towards anti-tumor immune reaction. TGF-β inhibits the expression of the transcription factors T-bet and GATA3, essential for the conversion of naive CD4+ T cells into Th1 and Th2 CD4+ T cells, respectively[58,59]. Conversely, TGF-β induces the differentiation of naive CD4+ T-cells into Tregs, inhibits the differentiation of naive CD8+ T cells to effector cells[60,61] and decreases perforin and IFN-γ expression, further impairing cytotoxic CD8+ T-cell activity[62].

Cell-mediated immune suppression

Immune evasion of tumor cells may be linked to altered antigen processing and presentation, deriving from HLA class I downregulation or from 2 microglobulin mutation/deletion[63]. HLA class I expression is essential for antigen presentation to CTLs and for tumor cell recognition by NK cells[64]. Tumor cell elimination by NK cells may be also impaired by decreased expression of the NKG2D ligand ULBP1 that correlates with early recurrence of HCC[65]. Another mechanism of HCC immune evasion from NK cell killing has been

ascribed to the impaired interaction between NKG2D and its stress-induced ligands MIC-A and -B, that are upregulated on tumor cells. In advanced HCC, tumor cells escape from NK-mediated immunosurveillance through shedding of MIC-A that induces downregulation of NKG2D thus affecting NK cell effector function[66].

Together with shared oncofetal and cancer-testis antigens, driver and passenger mutations occurring in the tumor cell genome can generate tumor-specific neoantigens that can contribute to tumor immunogenicity and represent potential immunotherapeutic targets[67]. Like viral antigens, TAAs undergo immune selective pressure that triggers the selection of resistant variants with survival advantage due to lower immunogenicity or immunosuppressive activity. The genetic instability of transformed cells favors this phenomenon of antigenic immunoediting[68]. Immune escape may also result from the secretion by HCC cells of immunosuppressive molecules as TGF-β, IL-10, indoleamine 2,3-dioxygenase (IDO), arginase, or from decreased co-stimulatory/increased inhibitory checkpoint signaling[69].

MDSCs represent a heterogeneous population of immature myeloid cells[70] that share suppressive functions[71,72] through different mechanisms: depletion of arginine[73] and cysteine[74] that are essential for T cell function, and release of reactive oxygen and nitrogen species that disrupt TCR signaling[75]. In addition, MDSCs promote tumor progression through neo-angiogenesis due to vascular endothelial growth factor (VEGF) production, and through enhanced tumor cell survival and dissemination[76].

In HCC MDSCs have been shown to inhibit NK cell function via NKp30 receptor[14] or through membrane-bound TGF-β[15] and to induce Tregs by IL-10 and TGF-β production[77]. A specific CD14pos HLA-DRneg/low MDSC subset increased in tumor tissue and peripheral blood of patients with HCC was implicated the induction of Tregs[77]. The multiplicity of this MDSC subset was reported as a negative prognostic factor for HCC recurrence after resection[78], radiation therapy[79], hepatic arterial infusion chemotherapy[80], as well as for tumor progression[81].

MDSCs are recruited by cytokines and chemokines secreted by tumor cells[72,82]. Senescent hepatocytes were shown to recruit immature MDSCs able to differentiate into macrophages through C-C motif chemokine ligand 2 (CCL2)-CCR2 signaling, thus preventing HCC initiation. However, in the presence of HCC, immature MDSCs do not differentiate thus contributing to the immunotolerant environment through NK-cell inhibition[83].

Kupffer cells (KCs), the liver resident macrophages, represent about 80% of the macrophages in the body[84] and contribute to the maintenance of liver immune tolerance through their anti-inflammatory function[85] exerted by upregulation of PDL-1 expression, downregulation of costimulatory molecules[86], secretion of IDO[87] and IL-10[88]. In human HCC, Kupffer cells in the peritumoral margin express higher levels of PD-L1 compared to non-tumorous liver, thus inhibiting CD8+ T cell effector function. Blockade of PD-1/PDL-1 interaction *in vitro* was able to restore T cell killing *in vitro*[89].

The HCC immune microenvironment induces the polarization of macrophages towards the M2 phenotype typical of the tumour-associated macrophages (TAMs). M2 macrophages are characterized by producing high levels of IL-10 that induce Treg expansion and impairs NK cell activation[90]. In addition, TAM promote tumor angiogenesis and dissemination[91,92]. A distinct subset of monocytes expressing TIE2 with enhanced pro-angiogenic properties has been described in peripheral blood and in tumor infiltrate[93-95]. In human HCV-related HCC this monocyte subpopulation was related to neo-angiogenesis and to prognosis[96].

Tregs are CD4+ T cells expressing CD25, CTLA-4, CD62L and FoxP3. Tregs exert inhibitory functions through multiple mechanisms, among which IL-2 depletion by CD25 (IL-2 receptor), competition with

CD28 by CTLA-4, CTLA-4-mediated downregulation of CD80 and CD86[97], expression of TGF-β and IL-10[98]. The recruitment of Tregs in HCC occurs via the CCR6-CCL20 axis[99] and CCL22 induction by tumor cell-secreted IL-1α[100]. In addition, FoxP3 upregulation and conversion of CD4+ T cells into Tregs may be fostered by poor stimulation of naive CD4+ T cells combined with TGF-β signalling by tumor cells[101].

In patients with HCC, FoxP3+ Tregs are increased both in peripheral blood[102,103] and in tumor tissue[31,103], and the abundance of tumour-infiltrating Tregs is associated with intra-tumoral macrophages[104]. Several studies support a negative correlation between Treg infiltrate and effector function of intra-tumoral CD8+ T cells[47,103] and a direct role of Treg infiltration over disease progression and overall survival[99,103-105].

HSCs play a role in HCC progression through release of hepatocyte growth factor[106] and induction of both MDSC[107,108] and Treg accumulation[109]. In addition, HSCs can also directly induce T cell apoptosis through PD-L1 expression[110]. Activated HSCs interact with monocytes inducing an immunosuppressive environment and contributing to poor prognosis in HCC[111].

NKT cells are a heterogeneous group of T lymphocytes sharing properties of both T cells and NK cells. NKT cells recognize glycolipid antigens via an invariant TCR α chain. The CD4+ iNKT-cells have been found to be enriched in intrahepatic malignant tumors[112]. Intra-tumoral CD4+ iNKT-cells produce Th2 cytokines that can inhibit expansion of tumor antigen-specific CD8+T-cells[112]. Consistent with this view, we observed that enrichment of iNKT cells in HCC infiltrate was predictive of shorter TTR[31].

Several other infiltrating or stromal cell types co-operate to the generation of immunosuppressive tumor microenvironment[113]. A population of PD-1-positive B cells has been identified in HCC. This cell subset was shown to suppress anti-tumor T cell response through PD-1-PDL-1 interaction and to promote disease progression[114].

LSECs express PDL-1 and contribute to the immunosuppressive environment by TGF-β-dependent induction of Tregs[115]. A subset of CD14+ DCs with suppressor function has been detected in patients with HCC. These DCs expressing high levels of CTLA-4 and PD-1 inhibit T-cell response through production of IL-10 and indoleamine-2,3-dioxygenase (IDO)[116]. Th17 cells are a IL-17-producing CD4+ T cell subset that plays an important role in the maintenance of mucosal barriers. Increased frequency of CCR4+CCR6+, but not CCR4-CCR6+ Th17+ cells was reported in peripheral blood from patients with HCC. The CCR4+CCR6+Th17+ cell subset was shown to impair CD8+ T cell effector functions[117]. Neutrophils release cytokines that contribute to the tumor microenvironment either promoting or inhibiting tumor progression[118]. In HCC, neutrophils have been shown to recruit macrophages and Tregs fostering tumor progression and resistance to sorafenib[119]. Tumor-associated fibroblasts (TAFs) are essential components of the HCC microenvironment and support tumor progression through the secretion of various cytokines and growth factors. HCC-associated TAFs inhibit NK-cell function by secreting prostaglandin E2 and IDO[120]. In addition, TAFs have been shown to release IL-6 and SDF-1a (CXCL-12), which induce MDSC generation and activation thus impairing anti-tumor immune response[121].

IMMUNOTHERAPEUTIC STRATEGIES

The scenario of the immune mechanisms operative in HCC is quite complex with liver intrinsic immunosuppressive environment associated with several mechanisms common to solid tumors. Physiologically, with the exception on anecdotal cases the tumor will escape, avoid, adapt to or overcome the immune mediated mechanism aimed at rejection of transformed cells. From the therapeutic perspective the problem has been approached by several strategies focusing on potentiation of different effector immune cells, tumor antigens made immunogenic, block of negative costimulatory pathways or immunosuppressive cells or soluble mediators.

Adoptive cell transfer

Adoptive transfer of autologous cytokine activated killer cells (CIK) has been one of the first immunotherapeutic approaches[122]. Anti-CD3 antibodies in the presence of IL-2, IL-1 and IFN-γ expand and activate *ex vivo* NKT-cells that are reinfused in the patient. This approach has been performed as adjuvant treatment in patients undergoing liver resection for HCC or percutaneous ablative treatments like percutaneous ethanol injection (PEI) or transarterial chemo-embolization (TACE). A systematic review of phase II and III studies conducted in patients undergoing CIK infusion either alone or associated with resection, PEI or TACE, showed a significant effect on overall and progression free survival[123]. More recently a randomized controlled trial of adjuvant CIK was conducted in patients undergoing curative liver resection showing a significant effect on TTR but no effect on DFS and OS[124]. Another randomized phase 3 study from Korea could demonstrate that patients receiving CIK post-resection, PEI or radiofrequency thermal ablation (RFA) had a significantly increased recurrence-free and overall survival[125]. Several other studies with similar methodological approach are ongoing in patients with HCC and other solid tumors.

Cancer vaccines

More than 15 years ago, discovery of TAAs raised enthusiasm on their possible use for vaccination strategies. Several different approaches have been employed from tumor lysates to individual epitopes associated with different adjuvants by parenteral route (subcutaneous, intradermal or intravenous), or intratumoral injection. Alternatively DCs pulsed with synthetic peptides or transfected with RNA vectors have been used to expand tumor-specific T-cell response. Target antigens for HCC have been cancer testis TAAs like MAGE, synovial sarcoma X breakpoint 2 (SSX-2) and NY-ESO-1, beside GPC-3, human telomerase reverse transcriptase (TERT), carcinoembryonic antigen (CEA) and AFP. Clinical trials have been conducted in patients with advanced or non-resectable HCC or as an adjuvant treatment in patients undergoing resection or RFA or TACE. Efficacy in these studies has been limited. Phase II studies with antigen-pulsed DCs[126], intradermal GPC-3 peptide[127], or intravenous tumor (HepG2 cell line) lysate-pulsed DCs[128] have been conducted showing partial response associated with antigen-specific T-cells responses in PBMCs in some patients. In particular, antigen-pulsed DCs vaccination[126] showed no tumor recurrence up to 24 weeks in 9 out of 12 treated patients. Phase I GPC-3 studies achieved their aims: safety, immunogenicity and dose finding, however phase II studies with GPC-3 aimed at relapse prevention after curative treatments (surgery or RFA) failed to achieve clinically relevant results[129,130]. Infusion of autologous DCs pulsed with lysate of HepG2 cell line was performed in a phase II trial in patients with advanced HCC. Clinical response (either stable disease or partial response) was shown in 28% of patients performing at least three infusions. Treatment was safe and antigen-specific immune response could be demonstrated in some patients[128].

A particular vaccination strategy has been conducted with an oncolytic, genetically modified vaccinia virus (JX-594) that has been injected in the tumor lesions of advanced HCC patients. The rational of this approach is the release of tumor antigens from oncolytic tumor cells destruction associated with local expression of granulocyte-macrophage colony stimulating factor (GM-CSF), an inserted gene of the genetically modified vaccinia. JX-594 has been tested in a dose finding study showing improved overall survival in patients receiving a higher infectious dose compared to lower dose[131]. A phase II trial failed to achieve survival advantage. In this trial however HCC patients were very advanced having progressed to sorafenib treatment. A phase III randomized clinical trial (NCT02562755) is now ongoing, comparing patients on sorafenib to patients undergoing three vaccination rounds followed by sorafenib treatment.

Table 1 represents a summary of completed clinical trials based on adoptive cell transfer and vaccines.

Cell therapy

More efficient adoptive cell transfer immunotherapeutic approaches, are represented by the CAR T-cell therapy which until now has been primarily used in hematologic malignancies. T cells are genetically engineered to express chimeric antigen receptors (CARs). Autologous engineered T cells are expanded *ex vivo* into the

Table 1. Adoptive cell transfer and vaccines for HCC immunotherapy

Therapeutic approach	Target	Phase	Study population	No. of patients	Results	Ref.
CIK	NA	II	Post-resection	76 + 74 controls	Improved RFS	[122]
CIK	NA	III	Post-resection	100 + 100 controls	Improved TTR	[124]
CIK	NA	III	Post-resection or RFA or PEI	114 + 112 controls	Improved RFS and OS	[125]
Peptide vaccine	GPC-3	I	Advanced HCC	11	Improved CTL response	[129]
Peptide vaccine	GPC-3	II	Post-resection or RFA	41	Improved RFS for patients with GPC-3 positive tumors	[130]
DC pulsed HepG2 protein lysate	Tumor antigens	II	Advanced HCC	35	PR 4%, SD 24%	[128]
DC pulsed AFP, MAGE-1 and GPC-3	Tumor antigens	I/II	Post-resection or RFA or PEI or TACE	12	Improved TTP *vs.* historical results	[126]
Oncolytic virus JX-594	Tumor antigens	II	Advanced HCC	30	Dose realated improved OS	[131]

HCC: hepatocellular carcinoma; RFS: recurrence free survival; TTR: time to recurrence; TTP: time to progression; OS: overall survival; RFA: radio-frequency ablation; PEI: percutaneous ethanol injection; TACE: transarterial chemo embolization: CTL: cytotoxic T lymphocytes; NA: not applicable

hundreds of millions and finally are infused in the patient. Third generation CARs are constituted of an immunoglobulin variable heavy chain (VH), a variable light chain (VL) connected to a transmembrane domain by a spacer and the transmembrane domain to 2 costimulatory molecules (e.g., CD27, CD28, 4-1BB, OX40) and CD3. This receptor when engaged can activate the effector cytotoxic T-cell, specifically redirected to the tumor antigen recognized by the VH and VL chains. As far as HCC, CAR-T have been designed with different specificities and phase I and phase I/II clinical trials are recruiting for patients with HCC or HCC and other solid tumors, targeting GPC-3, CEA and Mucin 1, cell surface associated (MUC-1)[132].

A different approach that engages T and NK-cells *in vivo* to direct them against tumor cells is represented by bispecific antibodies (BsAb). BsAb against HCC and other solid tumors have been generated with different specificities. One arm of antibody binds a tumor antigen [GPC-3, epithelial cell adhesion molecule (EpCAM), osteopontin, VEGF] and the second can activate cytotoxic T or NK cells binding CD3 or CD16. A phase 1 dose escalation trial with BsAb specific for GPC-3 and CD3 is ongoing (NCT02748837).

Another approach to generate tumor-specific immune cells is cloning and TCR transfection of T and NK cells that are *in vitro* expanded and reinfused in the patients. These redirected effector cells, differently from CAR-T or BsAb, recognize tumor epitopes in the context of specific HLA-class I molecules, but have advantage to recognize endogenously processed antigens, that is the case of many known epitopes from tumor associated antigens or neo-antigens from somatic mutations of the tumor-cell. In fact, cell therapies based on CARs and antibodies can only recognize conformational antigens expressed on the surface of transformed cancer cells. Redirect T-cells have been clinically tested in a patient that developed extra-hepatic metastasis after liver transplantation for HCC in HBV-related liver disease[133]. The tumor, but not the transplanted liver, expressed HBV antigens and autologous T-cells transfected with a TCR specific for HBsAg could expand *in vivo* and determine reduction of HBsAg serum levels.

Immune checkpoint inhibitors

The first clinical study on immune checkpoint inhibitors (ICIs) in HCC has been a phase II clinical trial targeting CTLA-4 in patients with advanced tumors in HCV chronic liver disease[134]. The study showed partial response in 17.6% of patients and a good safety profile. Transaminase flares were observed in some of the patients after the first anti-CTLA-4 administrations that however did not require any immunosuppressive intervention. Interestingly in this study an enhanced HCV-specific T-cell response associated with significant drop of HCV viremia was observed. Several other studies have started. The main target has been PD-1 and its ligand PD-L1 and recently FDA has granted accelerated approval for anti-PD-1 in patients that had been previously treated with sorafenib, based on the phase I/II Checkmate-040 study (that showed an overall

Table 2. Immuno check points inhibitors for HCC immunotherapy

Target	Number of patients	Trial	First line/Second line	Status	Results	Ref or study number
CTLA-4	20	Phase II	Adjuvant TACE and ablation	Completed	PR 17.6 %	134
CTLA-4	32	Pilot	Second	Completed	PR 15.6 %	137
PD-1	576	Phase I/II	Flirst and Second	Completed	PR 20 % (expansion) 15% (dose excalating)	135
PD-1	723	Phase III	First *vs.* sorafenib	Not recruiting active	NA	NCT02576509
PD-1	660	Phase III	First *vs.* sorafenib	Recruiting	NA	NCT03412773
PD-1	104	Phase II	Second	Not recruiting active	PR 15.4 %, CR 1%	NCT02702414
PD-1	408	Phase III	Second	Not recruiting active	NA	NCT02702401
PD-1	530	Phase III	Adjuvant SR and ablation	Recruiting active	NA	NCT03383458
PD-L1	114	Phase I	Second	Recruiting active	NA	NCT02519348
PD-L1 ± CTLA-4	440	Phase II	Second	Recruiting active	NA	NCT02519348
PD-L1 ± CTLA-4	1200	Phase III	First	Recruiting active	NA	NCT03298451

HCC: hepatocellular carcinoma; SR: surgical resecton; TACE: transarterial chemoembolization; NA: not available; PR: partial response; CR: complete response

response rate of 18.2% and acceptable safety profile)[135]. There was concern on possible immune mediated liver toxicity in patients with liver cirrhosis and chronic HBV or HCV infection. However, until now safety profile of ICIs has not shown to be different from what observed for melanoma and non-small cell lung cancer (NSCLC) and even if substantial transaminase flares have been described, patients coming off therapy for adverse events are in line with what observed for other cancers treated with anti-PD-1 or anti-PD-L1[136].

First line studies comparing ICIs to sorafenib treatment are ongoing in patients with advanced HCC: two studies from different companies, CheckMate-459 and NCT03412773 with anti-PD-1 and the HIMALAYA study testing the combined activity of an anti-PD-L1 and anti-CTLA-4. The adjuvant role of ICIs is also tested with anti-PD-1 versus placebo in patients with early stage HCC undergoing surgery or ablation evaluating relapse free survival as primary endpoint (NCT03383458). A study combining RFA, cryablation or TACE and-CTLA-4 in advanced HCC has been recently published[137]. Subtotal ablative treatments were given after the second anti-CTLA4 infusion. The study demonstrated feasibility and no dose-limiting toxicity of this therapeutic approach. Moreover 5/19 evaluable patients presented partial response. Interestingly pre and post-treatment biopsy showed an enrichment of CD3 and CD8 positive T-cells infiltrating the tumor after treatment that positively correlated with clinical response. Table 2 represents a more comprehensive list of completed and ongoing clinical trials with ICIs.

Until now it is not possible to understand which immune checkpoint is the most promising for HCC patients. Experience from other solid malignancies suggests that combining different ICIs may improve clinical response, given the increased risk of severe toxicities. Vaccination protocols combined with ICIs are tested in clinical trials, representing an alternative treatment strategy expanding tumor-specific T-cell populations *in vivo*[138].

Another immunotherapeutic approach that cannot be strictly considered an ICI is represented by an anti-TGFβRI (Galunisertib) that is expected to block the immunosuppressive and pro-tumorogenic effect of TGF-β. It has been tested in association with sorafenib in a phase II clinical trial (NCT01246986) showing a median overall survival of 17.9 months that represents an improved survival compared to sorafenib historical results.

PREDICTIVE BIOMARKERS

Although promising, the results of immunotherapy for HCC are far from optimal. Recent trials suggest that combined regimens with different ICIs would lead to higher rates of clinical response, but with increased

risk of immune-related adverse events. The development of biomarkers with acceptable predictive value will be instrumental to maximize the benefit of immunotherapy.

As previously described, the anti-cancer immune response is the results of multiple factors deriving from the antigenic characteristics of the tumor, the multiplicity and the phenotype of TAA-specific immune cells, the latter mainly dictated by the tumor microenvironment. From this perspective the use of a single analyte biomarker might not be sufficient to recapitulate the complex interplay between tumor biology and immune response. Immunostaining with anti-PD-L1 antibodies has been the first approach evaluated to predict the response to anti-PD-1 treatments. However, this marker was shown to be unreliable especially for its poor negative predictive value[139,140]. In addition, the intrinsic variability of immunohistochemistry together with the heterogeneity and dynamic nature of PD-L1 expression in tumor and immune cells raise concern about its adequacy to clinical standards[141].

Multianalyte profiles may represent promising tools for the accurate prediction of immunotherapy outcome. The response to PD-1 blockade has been related to the presence of immunogenic neoantigens arising from the active expression of viral genes or from increased tumor mutational burden[140,142]. According to this view, pembrolizumab (anti-PD-1) was approved by FDA in 2017 for the treatment of unresectable or metastatic solid tumors with mutations in genes for DNA mismatch repair (dMMR) or microsatellite instability (MSI), independently from the tissue of origin. However, dMMR or MSI are infrequent in HCC[143].

The multiplicity, composition, activity and location of tumor-infiltrating immune cells have been shown to represent prognostic markers in HCC[4-6,104]. A subset of HCCs characterized by an inflammatory gene signature has been detected in several studies[144-148]. A recent study identified in about 25% of patients an immune-specific molecular class of HCC including two distinct subtypes, characterized by a prevalent adaptive T-cell response and an exhausted immune response, respectively[146]. Interestingly, immune gene profiles suggesting active anti-tumoral response have been associated with longer time to recurrence[149,150].

Gene signatures may provide a global picture of the complex tumor immune landscape. This approach represents a tool for the discrimination of tumors with pre-existing immune infiltrate, more likely to respond to interventions aimed at overcoming inhibitory factors. In a recent paper a so-called "T cell-inflamed gene expression profile", containing IFN-γ-responsive genes related to antigen presentation, chemokine expression, cytotoxic activity, and adaptive immune resistance, was shown to be necessary, but not always sufficient, for clinical response to pembrolizumab in 10 tumor types[151].

The lack or low abundance of cellular infiltrate may indicate a defect in innate immunity or in immune cell trafficking and suggest alternative therapeutic approaches. Consistent with observations made in other tumors[152], a molecular profile of "immune exclusion" is associated with activated Wnt/β-catenin pathway signaling in HCC[149]. This suggests the potential of Wnt/β-catenin activation as a biomarker predictive of resistance to checkpoint inhibitors. As a future perspective, gene signatures integrating information about tumor cell mutational burden, presence and nature of the immune infiltrate, possibly at different investigational levels (genetic, genomic, epigenetic) would provide information for a comprehensive therapeutic stratification of HCC patients.

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