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New insights into the pharmacological, immunological, and CAR-T-cell approaches in the treatment of hepatocellular carcinoma

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ABSTRACT

The tyrosine kinase inhibitor (TKI) sorafenib continues to be the anchor drug in the treatment of advanced stage hepatocellular carcinoma (HCC). Other TKIs as well as immune checkpoint inhibitors (ICIs) have also been approved, however the response rates remain poor and heterogeneous among HCC patients, largely due to antitumor drug resistance. Studies aimed at identifying novel biomarkers and developing new strategies to improve the response to current treatment and to overcome drug resistance, are urgently needed. Germline or somatic mutations, neoantigens, and an immunotolerogenic state against constant inflammatory stimuli in the liver, are crucial for the anti-tumor response. A pharmacogenetic approach has been attempted considering germline polymorphisms in genes encoding for proteins involved in drug-targeted pathways. Single gene and comprehensive multi-gene somatic profiling approaches have been adopted in HCC to identify tumor sensitivity scores and immunogenic profiles that can be exploited for new biomarkers and innovative therapeutic approaches. However, the high genomic heterogeneity of tumors and lack of molecularly targeted agents, hamper the discovery of specific molecular markers of resistance to therapy. Adoptive cell therapy with chimeric antigen receptor redirected T (CAR-T) cells targeting specific tumor-associated antigens (TAAs) was proposed recently. The specificity of the chosen TAA, an efficient homing of CAR-T cells to the tumor site, and the ability of CAR-T cells to survive in the tumor microenvironment are central factors in the success of CAR-T therapy.

The current review describes the principal systemic treatments for HCC and the molecular evidence regarding potential predictive host and somatic genetic markers, as well as the emerging strategy of liquid biopsy for disease monitoring. Novel immunotherapeutic approaches for HCC treatment, including the use of ICIs and CAR-T, as well as strategies to overcome drug resistance, are discussed.

1. Introduction

Liver cancer is a global health problem, representing the sixth most frequent malignancy in terms of incident cases and the fourth most common cause of cancer-related death worldwide (Villanueva, 2019). Hepatocellular carcinoma (HCC) is the second most lethal tumor, with a 5-year survival rate of 18 % (Villanueva, 2019), and accounts for approximately 90 % of liver tumors (Llovet et al., 2016). HCC is a multifactorial disease that develops in a multistep process (Llovet et al., 2016; Villanueva, 2019). Most HCC cases arise on a background of liver damage, in which an ongoing liver injury and regeneration lead to inflammation and progressive liver fibrosis, predisposing patients to subsequent cirrhosis and neoplasia (Llovet et al., 2016; Villanueva, 2019). The major etiologies of HCC development include chronic infection with hepatitis B virus (HBV) or hepatitis C virus (HCV), liver cirrhosis, heavy alcohol intake, exposure to environmental/dietary carcinogens (i.e., aflatoxin B1), genetic and metabolic liver disease (i.e., hereditary hemochromatosis, non-alcoholic steatohepatitis), and other conditions of liver damage (Llovet et al., 2016; McGlynn et al., 2015). The prevalence of HCC increases with age and is greater among males than females (Llovet et al., 2016; McGlynn et al., 2015). In addition to environmental factors, recent insights into the biology of HCC have demonstrated that genetic and epigenetic abnormalities play an essential role in liver carcinogenesis: molecular changes occur at different evolutionary stages of HCC, and in most cases are induced by a specific etiological factor (Calderaro et al., 2019; Huang et al., 2018;

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Rebouissou and Nault, 2020; Toh et al., 2019). Genomic analyses of primary and recurrent HCC have indicated a complex mutational landscape with vast inter-tumor and intra-tumor heterogeneity that could account for HCC drug resistance, which represents a substantial challenge for improving the outcomes of patients with HCC (Liu et al., 2018).

Treatment decision-making in HCC is based primarily on the Barcelona Clinic Liver Cancer (BCLC) clinical classification. Early-stage cancers are potentially treatable by therapies with curative intent, such as liver transplantation, surgical resection, or local ablation with radiofrequency, whereas chemoembolization/radioembolization and systemic therapy represent the only therapeutic options for intermediate or advanced HCC (Llovet et al., 2016; Villanueva, 2019). Unfortunately, the overall prognosis for patients with HCC is dismal. Most patients with HCC are diagnosed at an advanced tumor stage, which precludes curative therapy, rendering systemic treatment the standard therapy. Even when improvements in surveillance programs permit an early diagnosis and curative treatment, almost half of all patients with HCC ultimately receive systemic therapies (Llovet et al., 2016; Vande Lune et al., 2018). Conventional chemotherapeutic agents (e.g., doxorubicin, fluoropyrimidines, platinum derivatives, and irinotecan) are minimally effective in HCC and do not improve patient survival (Llovet et al., 2018, 2016; Villanueva, 2019). As with various other human malignancies, the predominant underlying basis for the lack of activity of the above cytotoxic treatments is anticancer drug resistance. HCC is a particularly complex tumor with high intra-tumoral genomic and molecular heterogeneity, with a tendency to mutate under treatment-selective pressure. This is suggestive of the associated drug-resistance based on a plethora of mechanisms (Assaraf et al., 2019; Gacche and Assaraf, 2018; Kopecka et al., 2020; Marin et al., 2019; Taylor et al., 2015; Wijdeven et al., 2016; Zhang et al., 2020; Zhitomirsky and Assaraf, 2016). Thus, the development of novel strategies and anti-tumor agents that can surmount cancer drug resistance and achieve enhanced efficacy, is a major aim of current cancer research, including HCC (Assaraf et al., 2014; Bar-Zeev et al., 2017; Cao et al., 2020; Cui et al., 2018; Duan et al., 2019; Jiang et al., 2020b; Li et al., 2016; Livney and Assaraf, 2013; Narayanan et al., 2020).

HCC is rarely amenable to radiotherapy (Llovet et al., 2016; Villanueva, 2019). The introduction of targeted agents based on an improved molecular characterization of HCC has led to a new era of HCC treatment. In 2007, sorafenib, a small-molecule multikinase inhibitor, was the first systemic agent to increase the survival time of patients with advanced HCC (Llovet et al., 2008). The approval of sorafenib was followed by a long period of failure in testing novel drugs for the systemic treatment of HCC, but a recent explosion in additional agents with positive results in large phase III clinical trials has occurred. In particular, a number of other tyrosine kinase inhibitors (TKIs; i.e., regorafenib, lenvatinib, and cabozantinib), a monoclonal antibody targeting angiogenesis (i.e., ramucirumab), and immune checkpoint inhibitors (ICIs; i.e., nivolumab and pembrolizumab) have been approved as first-line (sorafenib and lenvatinib) or second-line (regorafenib, cabozantinib, ramucirumab, nivolumab, and pembrolizumab) treatment in patients with advanced HCC (De Mattia et al., 2019; Dhanasekaran et al., 2019; Villanueva, 2019; Zhu et al., 2019) (Fig. 1). Other small molecules, such as selective CDK4/6 inhibitors (palbociclib and ribociclib), are in earlier stages of clinical development for treating HCC, and the mesenchymal–epithelial transcription factor (MET) inhibitor tivantinib has not had positive results in phase III studies (Littman et al., 2015; Rimassa et al., 2018). Novel promising therapeutic strategies for HCC employing chimeric antigen receptor T (CAR-T) cells are also under investigation (Burga et al., 2015; Gao et al., 2014; Jiang et al., 2016; Katz et al., 2015; Zhang et al., 2016).

Though the introduction of targeted agents for the treatment of HCC has led to great advances in patient responses and survival, only a limited number of patients have achieved real and long-term benefits. The frequent drug resistance observed in clinical practice and significant inter-individual heterogeneity in therapy outcomes constitute important obstacles in liver disease management.

The present review describes the current principal systemic treatment approaches for HCC and critically discusses the pharmacogenomic evidence for potential predictive host and somatic genetic markers, as well as the emerging strategy of liquid biopsy for disease monitoring and overcoming drug resistance. The novel immunotherapeutic approaches for HCC, including the use of ICIs and CAR-T-cell therapies, as well as the main mechanisms underlying tumor/tumor microenvironment (TME)/immune system crosstalk, which limit their efficacy, are also discussed.

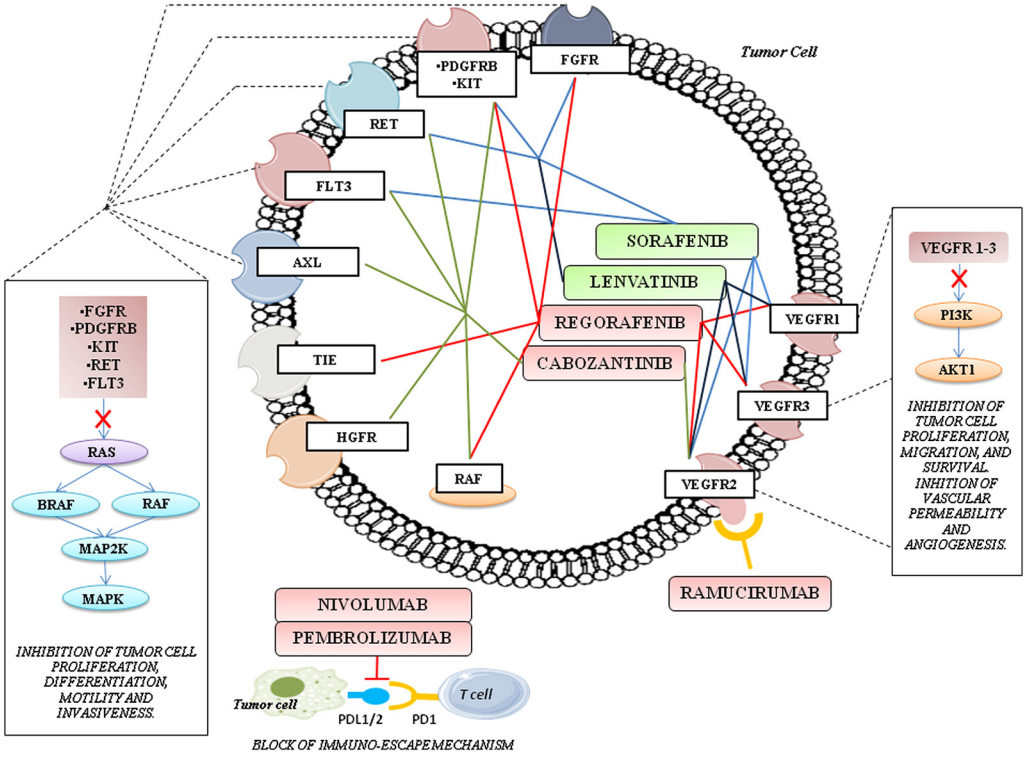
2. Liver cell crosstalk and HCC pathogenesis

HCC is characterized by the presence of vascular abnormalities with aberrant microvasculature generated by atherogenesis and capillarization (Liu et al., 2017). Different from normal liver, the vasculature is less dense with immature and abnormal leaky tumor vessels, resulting in interstitial hypertension, edema, and tumor hypoxia with necrotic regions (Liu et al., 2017). Consequently, hypoxia can re-stimulate angiogenesis and, eventually, tumor growth, resulting in a vicious cycle (Liu et al., 2017; Morse et al., 2019). The blood flow slows in the liver sinusoids, which constitutes the specific architecture of the liver (Jenne and Kubes, 2013). The liver sinusoids support the metabolic and immunological functions of the liver. Among the functions of the liver are the identification and clearance of microbes, microbe-associated molecular patterns (MAMPs), and damage-associated molecular patterns (DAMPs) (Jenne and Kubes, 2013). The execution of these tasks in the liver involves a large number of innate and adaptive immunity cells, particularly Kupffer cells, natural killer (NK) cells, natural killer T (NKT) cells, $CD4^+$ T cells, and $CD8^+$ T cells (Jenne and Kubes, 2013). In addition, several liver cells, including liver sinusoidal endothelial cells, hepatic stellate cells, and hepatocytes, play a role in the detection/recognition of pathogens, antigen presentation, and cytokine production (Heymann et al., 2015; Robinson et al., 2016). The balance between immune-tolerance and activation of the immune system in the liver depends on the above-mentioned elements (Heymann et al., 2015; Robinson et al., 2016).

In particular, immune tolerance is characterized by an immunosuppressive microenvironment determined by the levels of pro-inflammatory and anti-inflammatory cytokines. HCC pathogenesis originates from the deregulation of this well-tuned immunological network. In the presence of an unbalanced immunological state, the role of adaptive immune cells in the detection and elimination of HCC-transformed cells can be reduced because they are more inclined to exhaustion and tolerance (Heymann et al., 2015; Robinson et al., 2016). Thus, the TME consists of several factors that are extrinsic to cancer cells, such as various immune and stromal cells, components of the vasculature, extracellular matrix, and cytokines, all of which influence HCC pathogenesis and progression (Heymann et al., 2015; Robinson et al., 2016). In particular, regulatory T cells (Tregs) are capable of avoiding self-tolerance by suppressing the function of effector T cells through the secretion of several cytokines and direct contact, limiting inflammation. Moreover, infiltration of tumors by Tregs is a feature of many tumor types, including HCC (Chaudhary and Elkord, 2016). Myeloid-derived suppressor cells (MDSCs) also have regulatory function capable of promoting immune evasion and tumor growth (Khaled et al., 2013), facilitating tumor invasion, metastasis, and neoangiogenesis (Binnewies et al., 2018; Dysthe and Parihar, 2020; Finn and Ochoa, 2020; Gabrilovich, 2017; Jiang et al., 2020a; Kim et al., 2019; Mastio et al., 2019; Yang and Poon, 2008).

Tumor-associated macrophages (TAMs), particularly M2 macrophages, are able to stimulate tumor cell motility, angiogenesis, growth, and immune evasion (Noy and Pollard, 2014).

The cytokine status within the TME is responsible for both immunostimulatory and immunosuppressive effects, and are involved in



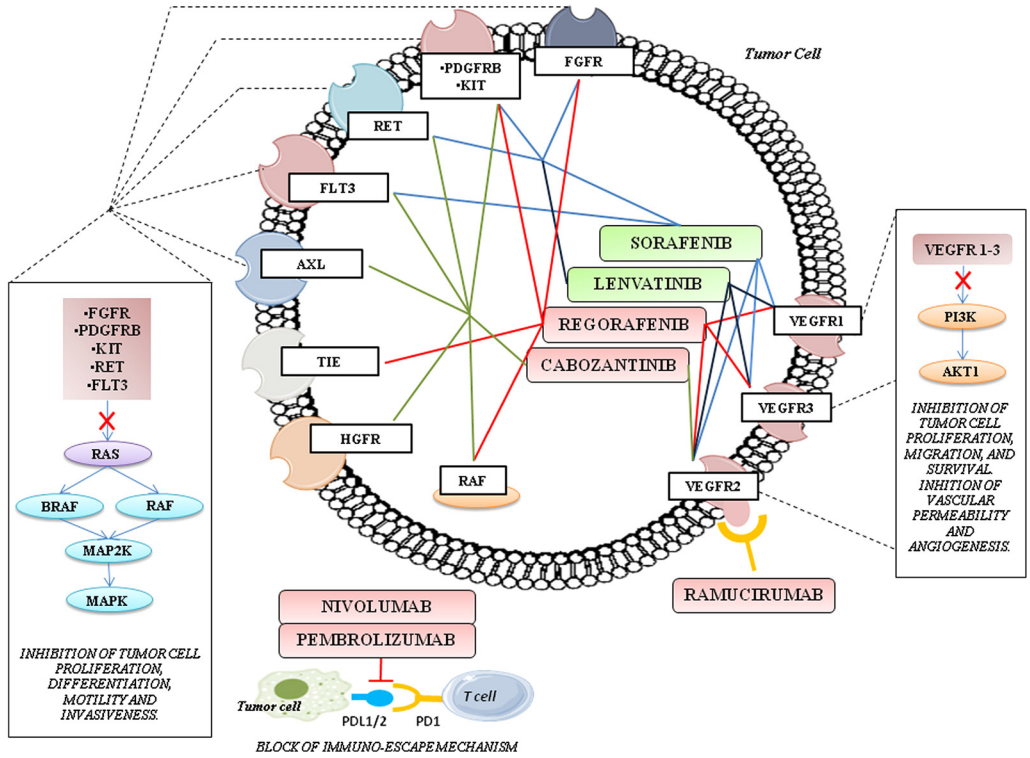


Fig. 1. Molecular targets and mechanisms of action of the drugs approved for systemic treatment of hepatocellular carcinoma. First-line agents are represented by an oval, and second-line agents by a square box. Abbreviations: AKT1, serine/threonine kinase 1; AXL, AXL receptor tyrosine kinase; BRAF, B-Raf proto-oncogene; FGFR, fibroblast growth factor receptor; FLT3, fms-related tyrosine kinase 3; HGFR, hepatocyte growth factor receptor; KIT, KIT proto-oncogene receptor tyrosine kinase; MAP2K, mitogen-activated protein kinase; PD1, programmed cell death protein-1; PDGFRB, platelet-derived growth factor receptor beta; PDL1/2, programmed cell death protein ligand; PI3K, phosphoinositide 3-kinase; RAF, serine/threonine-protein kinase; RET, RET proto-oncogene; TIE, tyrosine kinase with immunoglobulin-like and EGF-like domains; VEGFR, vascular endothelial growth factor receptor.

immune cell recruitment, activation, and proliferation. Immunosuppression is promoted by several chemokines capable of recruiting MDSCs and Tregs to the TME, including chemokine (C-C motif) ligand 5 (CCL5), CCL17, CCL22, interleukin (IL)8, and IL12. On the other hand, inhibition of C-C chemokine receptor type 4 (CCR4) diminishes trafficking of Tregs, promoting antitumor effects (Sugiyama et al., 2013). Moreover, cytotoxic T lymphocytes (CTLs) are recruited by IL9 and IL10, promoting anticancer activity (Moser, 2001; Paluskievicz et al., 2019). Transforming growth factor beta (TGF-β) signaling is involved in the modulation of various TME elements, influencing cell growth, differentiation, wound healing, apoptosis, and immuno-suppression. In particular, TGF-β is involved in the inhibition

of CTLs and the upregulation of Tregs.

Vascular endothelial growth factor (VEGF) acts as an immunosuppressive cytokine and is involved in the inhibition of the commitment of lymphoid progenitors, reducing the maturation of the T-cell lineage (Ohm et al., 2003). VEGF signaling is also capable of reducing trafficking and extravasation of CTLs into the TME, but promotes Treg infiltration of selective endothelium (Motz et al., 2014). VEGF also contributes to an increase in the expression of inhibitory receptors, contributing to CTL exhaustion (Voron et al., 2015).

3. Approved drugs for the systemic treatment of HCC

Four TKIs are now approved by the Food and Drug Administration (FDA) for the treatment of advanced HCC as first or second-line agents. Sorafenib is an orally administered small molecule that inhibits a number of serine/threonine and tyrosine kinases and related downstream pathways as outlined below. This drug represents the first targeted agent approved for the first-line treatment of HCC; it was approved in 2007 based on the positive results of the phase III SHARP trial. The trial found a median overall survival (OS) of 10.7 months for the sorafenib-treated group, compared to 7.9 months in patients who received placebo (Llovet et al., 2008). In 2017, another TKI, regorafenib, which has a mechanism of action similar to sorafenib, received FDA approval as a second-line agent for patients who tolerated but progressed on sorafenib. Approval was based on the findings of the phase III RESORCE trial, which reported an OS benefit of regorafenib compared to placebo (10.6 vs. 7.8 months) (Bruix et al., 2017). In 2018, levantinib, an additional member of the TKI family that selectively inhibits receptors related to pro-angiogenic and oncogenic pathways, such as vascular endothelial growth factor receptor 1–3 (VEGFR1–3), fibroblast growth factor receptor 1–4 (FGFR 1–4), platelet derived growth factor receptor alpha (PDGFRα), and rearranged during transfection (RET) and tyrosine-protein kinase (KIT) proto-oncogenes, was granted approval by the FDA as a first-line treatment for patients with unresectable HCC based on data from the REFLECT trial. This phase III trial comparing lenvatinib to sorafenib revealed the non-inferiority of lenvatinib compared to sorafenib for the primary endpoint OS (13.6 vs. 12.3 months, respectively) and significant improvement for secondary end-point progression-free survival (PFS) (Kudo et al., 2018). In 2019, cabozantinib, which can also block multiple oncogenic and angiogenic pathways implicated in tumor progression and metastasis, such as PDGFR, hepatocyte growth factor receptor (HGFR), VEGFR2, AXL receptor tyrosine kinase (AXL), RET, KIT, and fms-like tyrosine kinase 3 (FLT3), became an option for the second-line treatment of advanced HCC after sorafenib administration. The approval was based on the results of the CELESTIAL trial, which showed longer median OS (10.2 vs. 8.0 months) and PFS (5.2 months vs. 1.9 months) for carbozantinib compared to placebo (Abou-Alfa et al., 2018).

The presence of tumor-infiltrating lymphocytes (TILs) expressing programmed cell death protein-1 (PD-1) in HCC lesions and their correlation with patient outcome paved the way for immunotherapeutic approaches for the treatment of liver disease (Brizzi et al., 2016; Prieto et al., 2015; Shi et al., 2011; Siu et al., 2018). Among the novel class of ICIs that significantly improve the survival outcomes of patients with HCC, nivolumab and pembolizumab were approved recently as second-line agents, increasing the possible options for liver disease treatment.

Nivolumab is a fully humanized immunoglobulin G4 (IgG4) monoclonal antibody that exerts its activity by binding to the PD-1 receptor expressed on activated T cells, and blocking its interaction with programmed death ligand 1 (PD-L1) and PD-L2 on tumor cells. This leads to a down-regulation of the T-cell-promoted tumor immune-escape mechanism and restores the antitumor activity of T cells (Brahmer et al., 2015). Nivolumab represents the first ICI introduced as a therapeutic option for HCC. This drug was approved in 2017 by the FDA as a single-agent treatment for patients with advanced HCC who were previously treated with sorafenib based on the promising results of the CheckMate 040 trial (El-Khoueiry et al., 2017). This study demonstrated manageable safety of nivolumab for HCC treatment in terms of stable patient-reported outcomes, including indicators of health status and quality of life. In the subsequent multi-center phase III randomized CheckMate 459 trial, nivolumab demonstrated greater tolerability and clinically meaningful improvements in OS and both overall and complete response rates as first-line treatment compared to sorafenib (Yau et al., 2019b). However, the results did not achieve significance for the primary endpoints OS and time to progression (TTP) compared to sorafenib, and further studies will be required to determine the real efficacy of nivolumab in this therapeutic setting. In November 2018, another ICI, pembrolizumab, obtained accelerated approval by the FDA for the treatment of HCC patients who have previously received sorafenib based on positive findings in terms of an objective response from the open label phase II study KEYNOTE-224 (Zhu et al., 2018). Among assessable patients, this study recorded an objective response in 17 % of patients (1% complete response, 16 % partial response), achieving stable disease in 44 % of patients and progressive disease in the remaining 33 %. In the subsequent confirmatory study KEINOTE 240, the impact of pembrolizumab on OS and PFS was compared to placebo. Despite the advantage reported for pembrolizumab in terms of longer OS (13.9 vs. 10.6 months, hazard ratio [HR] 0.781, $P = 0.0238$) and PFS (3.0 vs. 2.8 months, HR 0.718, $P = 0.0022$), the results did not reach significance for the specified criteria (Finn et al., 2020). Currently, pembrolizumab is being investigated across multiple settings and lines of therapy for HCC through a broad scientific program of clinical trials. Pembrolizumab is a potent, selective, high-affinity humanized IgG4 monoclonal antibody that directly inhibits the binding of PD-1 to its ligands PD-L1 and PD-L2 (Siu et al., 2018). On March 10, 2020, the FDA granted accelerated approval for the combination of nivolumab and anti-cytotoxic T lymphocyte protein 4 (CTLA-4) ipilimumab for patients with HCC who have been previously treated with sorafenib. The efficacy of the combination was investigated in cohort 4 of CHECKMATE-040, patients with HCC who progressed on or were intolerant to sorafenib. The overall response rate was 33 %, with 4 complete responses and 12 partial responses. Response duration ranged from 4.6 to 30.5 months, with 31 % of responses lasting at least 24 months (Yau et al., 2019a).

Ramucirumab is the most recent drug introduced as a therapeutic option for advanced HCC. This agent is a fully human IgG1 monoclonal antibody that directly and selectively binds the extracellular domain of VEGFR2 with high affinity and blocks the interaction with its natural ligands VEGF-A, VEGF-C, and VEGF-D. As VEGF molecules are secreted by tumor cells to promote angiogenesis, the binding of ramucirumab to VEGFR-2 leads to the inhibition of ligand-induced proliferation and the migration of endothelial cells (Syed, 2020). In May 2019, ramucirumab received approval from the FDA as a single agent for HCC in patients who have an alpha-fetoprotein (AFP) level ≥ 400 ng/ml and were previously treated with sorafenib. Approval was based on positive findings from the randomized, double-blind phase III REACH-2 trial, which indicated that ramucirumab significantly improved OS and PFS relative to placebo in this specific population (Zhu et al., 2019). Ramucirumab also represents the only drug specifically tested and approved for patients presenting with a high level of AFP, a condition strictly related to aggressive disease and poor prognosis (Zhu et al., 2019).

4. Genetic markers and efficacy of systemic treatment

4.1. Host pharmacogenetic markers

In the context of discovering predictors of the response to systemic treatment in HCC, genetic polymorphisms, with their well-established role in liver carcinogenesis (De Mattia et al., 2017a, b), could be important and contribute, in combination with clinical and molecular parameters, to stratifying patients according to different therapeutic

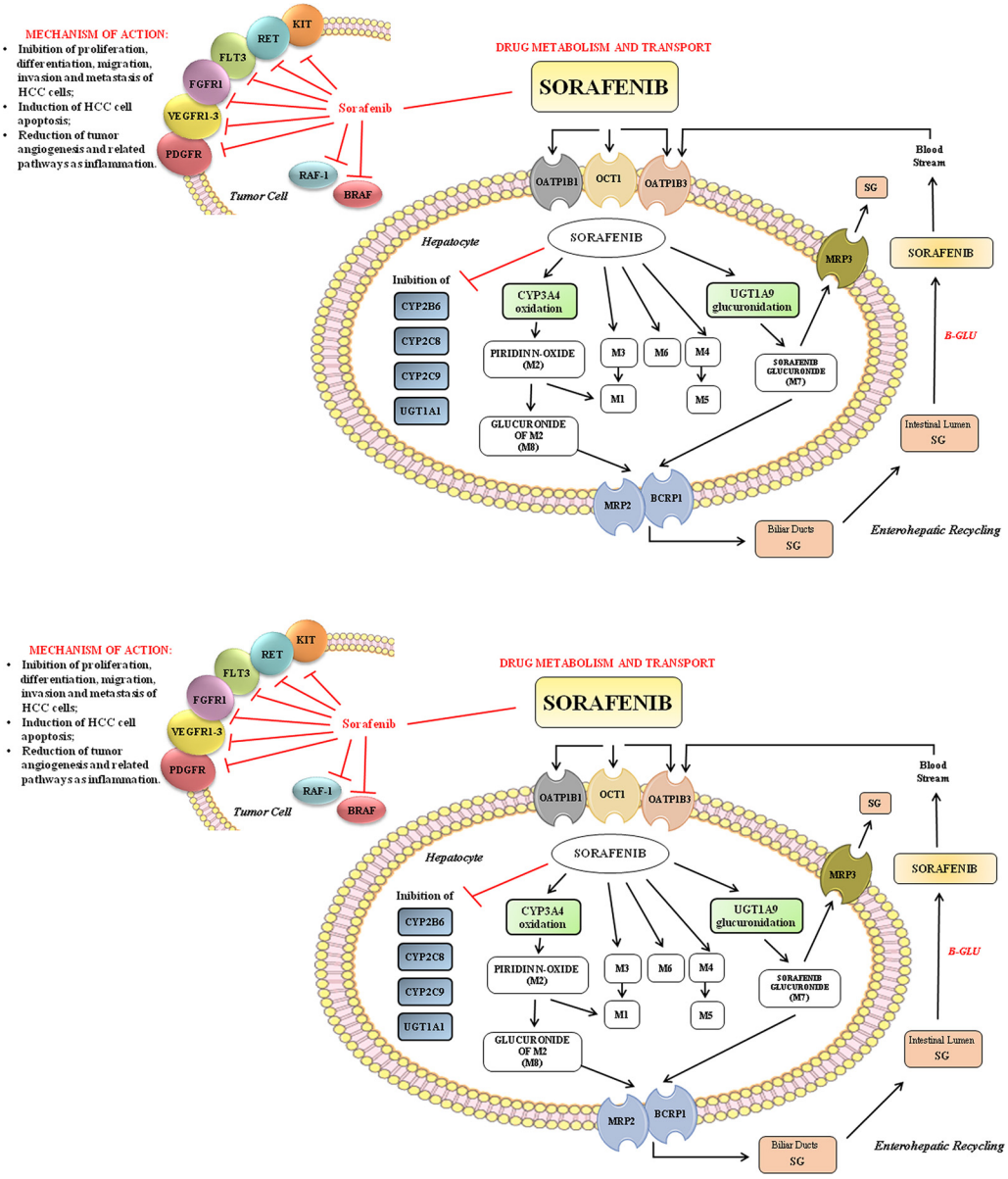


Fig. 2. Metabolism, transport, and molecular targets of sorafenib. The anticancer activity of sorafenib is carried out via the inhibition of some membrane serine/threonine and tyrosine kinases (i.e., vascular endothelial growth factor receptor [VEGFR1-3], platelet-derived growth factor receptor [PDGFR], fibroblast growth factor receptor 1 [FGFR1], KIT proto-oncogene receptor tyrosine kinase [KIT], ret proto-oncogene [RET], fms-related tyrosine kinase 3 [FLT3]) and downstream oncogenic Raf signaling members (i.e., the serine/threonine-protein kinases Raf-1 and B-Raf), impacting multiple tumor-related signaling pathways. Sorafenib is metabolized mainly in the liver through two pathways: phase I oxidation (i.e., cytochrome P450 3A4 [CYP3A4]) and phase II conjugation (i.e., UDP glucuronosyltransferase 1A9 [UGT1A9]) (Gong et al., 2017; Keating, 2017). Eight metabolites (M1–M8) have been identified in addition to the glucuronide form (SG). Sorafenib also inhibits the activity of some enzymes, including the cytochrome isoforms CYP2B6, CYP2C8, and CYP2C9, and the UDP glucuronosyltransferase UGT1A1, with potential drug interactions. Membrane transport of sorafenib and its metabolites is carried out by ATP-binding cassette (ABC) and solute carrier (SLC) transporters, many of which have not been identified. Enterohepatic recirculation has also been suggested (Vasilyeva et al., 2015). Briefly, after oral administration, sorafenib enters the hepatocytes via hepatic uptake transporters, including organic anion transporter family member 1B (OATP1B1 and OATP1B3) and cation transporter-1 (OCT1). After CYP3A4- and UGT1A9-mediated metabolism, SG is extruded from the hepatocytes into the bile, mainly by the multidrug resistance protein 2 (MRP 2/ABCC2) and breast cancer resistance protein (BCRP/ABCG2). A considerable fraction of intracellular SG is secreted back into the blood by some sinusoidal transport mechanisms, including MRP3 (ABCC3). From the circulation, downstream hepatocytes can efficiently take up SG again via the OATP-type carriers, resulting in only low SG concentrations reaching the general circulation. Once secreted into the bile, SG enters the intestinal lumen, where it can be a substrate for bacterial β-glucuronidases that regenerate the parental drug sorafenib (Gong et al., 2017; Keating, 2017; Vasilyeva et al., 2015).

outcomes in terms of drug efficacy. In the last few years, several pharmacogenetic studies have been published focusing on the orally administered multi-target TKI sorafenib, which has been used longer than other agents in HCC treatment (De Mattia et al., 2019).

The metabolism, transport, and mechanism of action of sorafenib is described in Fig. 2 (Gong et al., 2017; Keating, 2017; Vasilyeva et al., 2015). Sorafenib exerts its anticancer activity by inhibiting some serine/threonine and tyrosine kinases (i.e., VEGFR1–3, PDGFR, FGFR1, KIT, RET, FLT3) and factors involved in downstream oncogenic Raf signaling (i.e., Raf-1 and B-Raf), impacting multiple tumor-related signaling pathways (Fig. 2) (Gong et al., 2017; Keating, 2017). Data from in vitro analyses and animal models have demonstrated that sorafenib acts by repressing the proliferation of HCC cells and tumor growth, inducing HCC cell apoptosis, and reducing tumor angiogenesis and related pathways (e.g., inflammation) (Gong et al., 2017; Keating, 2017). Mitogen-activated protein kinase (MAPK)-independent induction of apoptosis and immunomodulatory effects have also been described as other mechanisms implicated in the activity of sorafenib. Thus, intrinsic and acquired resistance to this targeted agent is a complex and multifaceted phenomenon for which the underlying mechanisms are not completely defined.

A number of pharmacogenetic studies investigating the role of inherited genetic variability in determining the response to sorafenib in advanced HCC patients have focused on the VEGF/VEGFR cascade and analyzed the functionally relevant polymorphisms in genes encoding VEGF and VEGFR (Faloppi et al., 2016; Scartozzi et al., 2014; Zheng et al., 2014). The retrospective multicenter study ALICE-1 (Angiogenesis LIver CancEr) (Scartozzi et al., 2014) indicated that the alleles *VEGFA* rs2010963-C and *VEGFC* rs4604006-T are independent predictors of longer PFS and OS in the multivariate analysis. Combining these two markers further improved patient stratification. The favorable alleles *VEGFA* rs2010963-C and *VEGFC* rs4604006-T were also significantly associated with a better objective response. These findings were confirmed in the subsequent and larger multicenter study ALICE-2

(Faloppi et al., 2020) in which harboring the two favorable alleles, *VEGFA* rs2010963-C and *VEGFC* rs4604006-T, compared to only one or none resulted in three populations with different TTP and OS. Although the functional effects of *VEGFA*-rs2010963 in the 5'-UTR and the intronic *VEGFC*-rs4604006 variants have not yet been fully characterized, these polymorphisms likely affect the level of circulating VEGF, interfering with the mechanism of action of sorafenib and its anti-angiogenic activity. Another study focused on polymorphisms in the gene (i.e., kinase insert domain receptor [*KDR*]) encoding the receptor VEGR2, the dysfunction of which correlates with the decreased anti-apoptotic effects of VEGF, among other vascular alterations (Zheng et al., 2014). At the multivariate level, the rs1870377-AA genotype was associated with longer TTP and OS, and the rs2071559-T allele with longer OS. A positive effect of the rs1870377-AA genotype on the response to treatment was also suggested. Both the missense rs1870377 (Gln472His) variation, located in the fifth NH_2-terminal Ig-like domains within the extracellular region, and rs2071559 located in the promoter region have been reported to affect VEGFR2 functionality and/or expression (Zheng et al., 2014).

Other genes belonging to VEGF-dependent pathways were studied for their potential implication in sorafenib resistance. In particular, genes harboring genetic polymorphisms in endothelial nitric oxide synthase (NOS3, encoded by *eNOS*), hypoxia inducible factor 1 subunit alpha (HIF1α, encoded by *HIF1A*), and angiopoietin-2 (Ang-2, encoded by *ANGPT2)* demonstrated a predictive/prognostic value in HCC patients treated with sorafenib (Casadei Gardini et al., 2016; Faloppi et al., 2020; Marisi et al., 2019). The Italian multicenter ePHAS study (Casadei Gardini et al., 2016), which adopted a training/validation study design, focused on three functionally relevant polymorphisms in *eNOS*. An alteration in NOS3 activity could impact the efficacy of sorafenib given the direct correlation between activation of the VEGF signaling pathway and stimulation of the vasodilator nitric oxide. The study found a detrimental impact of a specific *eNOS* haplotype (HT1:T-4b) on PFS and OS based on the combination of an rs2070744 T-to-C substitution in the 5'-UTR and the intronic VNTR 27bp 4a/4b polymorphism. Patients homozygous for the *eNOS* haplotype HT1 had approximately 5-fold higher risk of disease progression and 2.5-fold higher risk of death than those with other haplotypes. These findings were confirmed recently by another analysis of the same group when updating the follow-up of the previously described case series (Marisi et al., 2019). An impact of an *eNOS* variant (rs2070744) on the disease control rate (DCR) was also preliminarily suggested (Marisi et al., 2019). The two polymorphisms, rs2070744 and VNTR 27bp 4a/4b, were described to directly affect gene transcription efficiency, resulting in higher NOS3 protein activity and increased basal NO production, which could alter the activation of VEGF signaling and contribute to sorafenib resistance (Marisi et al., 2019). Another multicenter study, ALICE-2 (Faloppi et al., 2020), focused on polymorphisms in the *HIF1A* gene; the encoded HIF1α transcription factor is stabilized under hypoxic conditions and up-regulates VEGF expression by binding to the *VEGFA* promoter, resulting in enhanced angiogenesis. This study showed in the multivariate analysis that the *HIF1A* rs12434438-GG genotype is a predictor of poor therapy outcome in terms of shorter PFS and OS, independent from VEGF markers (i.e., *VEGFA* rs2010963 and *VEGFC* rs4604006). An additional clinical study (Marisi et al., 2019) reported positive findings for genetic markers in another key angiogenic factor, Ang-2 (encoded by *ANGPT2*). By binding to its receptor Tie2, Ang-2 cooperates with the VEGF pathway in regulating angiogenesis and maintaining normal physiological vascular functions. This protein is also suggested to contribute to determining tumor aggressiveness and metastatic phenotype. The analysis identified three ANGPT2 polymorphisms that were significantly associated with OS and/or PFS: rs55633437, rs3020221, and rs1961222. Among these polymorphisms, *ANGPT2*-rs55633437 remained significant after multivariate regression. The rs55633437-T allele was associated with lower PFS and OS, higher risk of extra-hepatic spread, and lower percentage of DCR with the GG-genotype. The functional impact of the synonymous variant rs55633437 (Thr186Thr) is still unknown, but this polymorphism may alter the Ang-2 expression levels, as suggested by Marisi et al. (2019). An exploratory haplotype analysis identified an *ANGPT2* haplotype characterized by rs3739392, rs3739391, and rs3739390 variants that is significantly associated with worse OS and PFS. These three variants, located in the 5'-UTR region of the gene, was predicted by bioinformatics tools to affect binding to transcription factors and protein synthesis (Marisi et al., 2019). Moreover, by combining non-synonymous *ANGPT2* and *eNOS* variant profiles, patients exclusively characterized by variants in the regulatory region of *eNOS* had a better prognosis than patients affected by variations in the 5′-UTR of *ANGPT2* (Marisi et al., 2019).

The vast majority of available pharmacogenetic studies have adopted a candidate gene or pathway-based approach. Alternatively, Lee et al. (2015) performed whole genome sequencing (WGS) to identify novel markers associated with the response to sorafenib in unresectable patients with HCC. The study was based on a preliminary genome analysis of extreme phenotypes (good and poor responders to sorafenib) to find potential predictive markers, and on a subsequent validation in a larger cohort of patients. The most relevant finding of the study was the identification of non-synonymous genetic variants in the membrane transporter gene *SLC15A2* as the most important predictors of the response to sorafenib. The T allele of the *SLC15A2* rs2257212 (Leu350Phe) polymorphism was related to a significantly longer PFS than the CC genotype. In vitro functional analysis indicated that hepatic cells harboring the TT genotype had a better response to sorafenib than cells with the CC genotype; structural changes, rather than changes in expression, were suggested to be the cause of the functional alteration. These findings underline the utility of a WGS-based approach to discover potential novel predictors of sorafenib efficacy.

Regarding the other approved agents recently introduced for the treatment of patients with HCC, to the best of our knowledge, no studies have investigated the correlation between host genetic polymorphisms and therapy efficacy in patients with HCC, and only sparse data are available in other tumor settings (De Mattia et al., 2019). However, regorafenib is characterized by a metabolism and mechanism of action similar to sorafenib, and the same angiogenesis-related targets highlighted for sorafenib could also be predictors of the response to regorafenib. This hypothesis is supported by preliminary results from recent studies performed in other cancer settings, such as colorectal cancer, in which regorafenib has been used for a long time (De Mattia et al., 2019).

Concerning the ICIs nivolumab and pembrolizumab, a few preliminary hypothesis-generating findings have been derived from other tumor settings, such as lung cancer (Nomizo et al., 2017; Yeo et al., 2017). This evidence suggests a role of polymorphisms in *PD-1/PD-L1* and related pathway genes in the response to ICIs by altering their mechanism of action. Additional studies are recommended to test the usefulness of *PD-1/PD-L1*-related markers in the setting of HCC. In this context, *PD-L1* rs2297136, rs4143815, and rs17718883 variants have recently been demonstrated to be associated with HCC risk and prognosis (Yeo et al., 2017; Zou et al., 2019). These data further reinforce the importance of investigating the role of *PD-L1*-related polymorphisms as predictive markers of the outcome in patients with HCC treated with ICIs.

4.2. Somatic tumor mutations

Despite improvements in understanding the molecular mechanisms underlying HCC development and progression (Ogunwobi et al., 2019), reliable prognostic biomarkers discriminating tumors that are responsive and non-responsive to systemic treatment have not yet been identified. In recent years, the rapid technological advances in genetics have permitted a deeper understanding of the mutational profile of

tumor tissue, leading to more effective molecular and clinical classification of the same tumor in terms of both disease prognosis and sensitivity to treatment. In addition, somatic characterization of tumors has been shown to allow the identification of potential molecular targets for drug therapy and possible markers of drug resistance or sensitivity to treatment.

A number of studies have focused on the somatic HCC profile, including single nucleotide variations, insertion/deletions, structural variations, and copy number alterations, by exploring the mutational status of liver tissue samples via sequencing (Dhanasekaran et al., 2019; Huang et al., 2018; TCGA, 2017). These analyses reported recurrent mutations in 12 genes: tumor protein p53 (*TP53*), catenin beta 1 (*CTNNB1*), axis inhibition protein 1 (*AXIN1*), albumin (*ALB*), AR-rich interaction domain 2 (*ARID2*), AT-rich interaction domain 1A (*ARID1A*), ribosomal protein S6 kinase A3 (*RPS6KA3*), apolipoprotein B (*APOB*), retinoblastoma associated protein (*RB1*), cyclin-dependent kinase inhibitor 2A (*CDKN2A*), low-density lipoprotein receptor-related protein 1B (*LRP1B*), phosphatase and tensin homolog (*PTEN*). The tumor suppressor *TP53* and WNT pathway oncogene *CTNNB1* are the most frequently mutated genes. Genetic alterations in the catalytic telomerase reverse transcriptase (*TERT*) gene have also been recognized as frequent events in HCC. Distinct molecular subtypes of HCC based on the somatic profile have also been defined, and in some cases were related to different clinical and histological features, tumor aggressiveness, disease recurrence risk, patient prognosis, and therapeutic outcome (Chang et al., 2019; Ding et al., 2019; Harding et al., 2019; Nishida et al., 2018; Schulze et al., 2015; Woo et al., 2011). Of particular interest is the study by Nishida at al. (2018), who performed comprehensive genetic, epigenetic, and chromosomal analyses in more than 100 HCC tissue samples, identifying molecular features related to tumor aggressiveness. In particular, the HCC subtype characterized by the *TP53* mutation, high fractional allelic loss (FAL), and global hypomethylation was associated with aggressive tumor characteristics, such as vascular invasion, whereas the *CTNNB1* mutation was a feature of the less aggressive phenotype. This molecular scoring system was also shown to predict the emergence of metastatic recurrence after curative treatment, such as liver transplantation. The number of molecular risk factors (i.e., *TP53* mutation, high FAL, significant global hypomethylation, absence of *CTNNB1* mutation) was associated with shorter recurrence-free survival in liver transplantation patients ($P = 0.0090$, log-rank test). These results were confirmed in a cohort of resected HCC cases from The Cancer Genome Atlas (TCGA; $P = 0.0076$). The identified molecular risk score could have important clinical implications for optimizing the administration of systemic therapy after curative treatment and represents a good marker for more precise selection of HCC patients with undetectable tumor dissemination and subsequently increased risk of metastatic recurrence. These patients require systemic therapy with novel TKIs and ICIs that could improve survival in such HCC cases.

Another recent investigation focused instead on the clinical utility of somatic profiling in intermediate and advanced stages of HCC in which tumor genomic biomarkers of drug response have not yet been identified (Harding et al., 2019). This analysis included 127 patients with HCC who received systemic therapy, among other therapeutic interventions, prospectively genotyped by next-generation sequencing (NGS) with a panel of 341 or more cancer-associated genes. The study indicated that, in the sorafenib-treated group (n = 81), patients whose tumors harbored activating mutations in the oncogenic phosphoinositide 3-kinase (PI3K) – mammalian target of rapamycin (mTOR) pathway had a poor prognosis in terms of lower DCR (8.3 % vs. 40.2 %) and shorter median PFS (1.9 vs. 5.3 months) and OS (10.4 vs. 17.9 months) compared to those without such mutations. In the ICI-treated group (n = 31), the presence of activating Wnt family member 1 (WNT)/β-catenin alterations were associated with innate resistance to therapy (clinical benefit 0% vs. 53 %), as well as a significantly shorter median PFS (2.0 vs. 7.4 months) and numerically shorter median OS (9.1 vs. 15.2 months) compared to non-mutated tumors. Moreover, this work indicated that 24 % of patients harbored potentially druggable alterations, e.g., mutations in *MTOR, HGFR,* tuberous sclerosis 1/2 (*TSC1/2*), fibroblast growth factor 19 (*FGF19*), isocitrate dehydrogenase 1 (*IDH1*), or other genes that are targets for currently available FDA-approved drugs, or agents in active clinical development whose identification could create new therapeutic opportunities (Ding et al., 2019; Liu et al., 2018; Lu et al., 2016). An additional preliminary study investigated the potential mutational pattern associated with the response to regorafenib (Teufel et al., 2019). In particular, tumor tissues from patients with HCC who received regorafenib in the RESORCE trial (7 responders and 10 non-responders) were characterized by NGS with a panel of 315 cancer-related genes. Mutational analysis revealed 49 somatic variants in 27 oncogenes or tumor suppressor genes, the most frequent of which were aberrations in the promoter region of *TERT* and mutations in *TP53*. Somatic alterations in the PI3K pathway, i.e., *PTEN*, phosphatidylinositol 3-kinase (*PIK3CA*), or *TSC1/2*, were equally distributed between responders and non-responders. In contrast, mutations in *CTNNB1,* encoding a member of the Wnt pathway, β-catenin, were more frequent in regorafenib non-responders (mutations detected in 3 of 10 non-responders) compared to responders (mutations detected in any of the 7 responders), as they occurred only in patients with disease progression upon regorafenib treatment. *VEGFA* gene amplification was detected only in one patient, a regorafenib responder. These hypothesis-generating findings suggest potential somatic mutations that could be candidate markers for predicting the response to regorafenib treatment in HCC and that warrant further investigation. Of interest is a very recent case report based on a patient with advanced HCC and treated with cabozantinib and nivolumab (Yang et al., 2020). Characterization of the tumor tissue by NGS of 450 tumor-related genes revealed that *RET* gene amplification, high tumor mutational burden ($\geq$10 mutations per megabase), and PD-L1 expression are potential markers of the response to combination cabozantinib and nivolumab in terms of an extraordinary longer PFS (25 months). This observation, in addition to suggesting the feasibility of combining more targeted agents, emphasizes the need to adopt multiple detection technologies, including NGS and immunohistochemistry, to define a more complete predictive score of the response to HCC therapy.

Despite the great opportunities recently presented by somatic characterization of HCC to optimize systemic treatment of this disease, some limitations of clinical practice must be considered. Genomic analyses of recurrent and primary tumors have revealed that HCC is one of the most heterogeneous tumors. The well-documented high intratumor (within a tumor) and inter-tumor (tumor by tumor) heterogeneity, as well as heterogeneity between primary and recurrent tumors, makes the development and administration of systemic targeted therapies challenging (Ding et al., 2019; Liu et al., 2018; Lu et al., 2016). The molecular heterogeneity of HCC has been indicated to significantly contribute to drug resistance and tumor relapse, acting as a considerable obstacle to improving the outcomes of HCC patients. In this context, a single tumor biopsy specimen is inadequate to represent the high molecular heterogeneity of HCC and makes biomarker identification, which is critical for optimized administration of targeted agents, rather difficult. In many cases, the HCC tissue was obtained from a primary tumor many years before the development of advanced disease, when patients were treated with systemic therapy. These “advanced tumors” can accumulate additional molecular alterations and become molecularly distinct from the “primary tumor”, making the somatic signature obtained from primary tissues inadequate for predicting the drug response of advanced tumors. An improved understanding of the significance of HCC heterogeneity and molecular alterations in HCC over time, as well as the development of novel strategies (i.e., biomarker enrichment, adaptive design strategies, multi-region and longitudinal tumor sampling) are required to implement successful personalized, targeted therapy. Another limitation to be considered is the scarce availability of biopsies of liver tissue, mainly

in the setting of intermediate-advanced HCC. In contrast to other tumors, biopsies are rarely carried out in HCC, as they are usually not required for diagnosis. Therefore, the somatic mutational profile is commonly not assessable in clinical practice because of the lack of either biopsy or surgical tissue, as only systemic treatments are recommended for late-stage HCC.

4.3. Liquid biopsy and disease monitoring

Considering the lack of representative HCC tumor tissues and the poor availability of biopsies or surgical samples, the application of emerging strategies based on circulating cell free DNA (ccfDNA) and its variable tumor-derived fraction (ctDNA) could be particularly promising in the context of HCC (Labgaa et al., 2018; Mezzalira et al., 2019; Ng et al., 2018). Liquid biopsy is emerging as an opportunity to perform a non-invasive analysis of somatic molecular alterations, as the ctDNA has been reported to carry genetic information consistent with tumor cells (Labgaa et al., 2018; Mezzalira et al., 2019; Ng et al., 2018). In particular, the pilot study by Labgaa et al. (2018) investigated mutations in circulating DNA and their correlation with those detected in the tumor tissue by multi-regional sampling in eight patients with HCC treated by surgical resection. The analysis was performed by ultra-deep targeted sequencing of all exons of the 58 most frequently mutated genes in HCC. The study confirmed the tumor origin of the mutations found in the circulating DNA in the plasma, providing the first strong evidence of the release of HCC-derived DNA fragments into the bloodstream. Similar findings were found by Ng et al. (2018), who analyzed biopsies from the primary tumor tissue and plasma of 30 prospectively recruited, systemic treatment-naive HCC patients using deep sequencing targeting 46 HCC driver genes and mutation hotspots. Interestingly, among the patients with high disease burden (large tumor or metastasis), who are most likely ineligible for surgical resection, ccfDNA and tumor DNA profiling captured similar proportions of somatic mutations.

Other recent pilot studies have demonstrated that characterization of the somatic signature through ctDNA analysis could also reveal important information regarding tumor heterogeneity and its dynamic evolution over time (Cai et al., 2017; Ikeda et al., 2018). The results indicated that ctDNA could overcome the intra-tumor heterogeneity present in patients with advanced HCC during treatment and follow-up; ctDNA could also track, in real-time, the therapeutic responses in longitudinal monitoring.

Some case-control studies (Huang et al., 2012; Oh et al., 2019; Park et al., 2018; Piciocchi et al., 2013; Ren et al., 2006; Tokuhisa et al., 2007) have focused on evaluating the variation in the amount of ccfDNA in the plasma or serum of healthy and cancer patients in order to establish a cut-off value for discriminating between the two groups. These analyses adopting different methodological approaches for measuring ccfDNA levels demonstrated that the quantity of ccfDNA detected in cancer patients with both early-stage or advanced disease was significantly higher than in healthy or non-cancer patients (i.e., cirrhotic patients or chronically HBV/HCV infected patients without HCC).

In the context of early-stage HCC treated by surgical resection, high circulating tumor DNA concentrations are associated with the presence of larger and more invasive tumors, as well as shorter OS, DFS rates, and recurrence (Huang et al., 2012; Piciocchi et al., 2013; Ren et al., 2006; Tokuhisa et al., 2007). Two recent studies focused on the potential of quantitative circulating DNA analysis in therapeutic settings other than surgery, such as radio-chemotherapy (Park et al., 2018) and advanced liver disease receiving systemic treatment (Oh et al., 2019).

An increasing number of studies have started to support quantitative ccfDNA evaluation with qualitative analysis (i.e., somatic mutational profile, altered fragmentation, aberrant methylation pattern) of specific tumor DNA features in plasma. A number of recent publications (An et al., 2019; Cai et al., 2019; Liao et al., 2016) focused on investigating qualitative ccfDNA alterations in terms of sequence variation, adopting either a targeted approach or whole genome or exome sequencing approaches.

Most published studies have been performed on HCC patients with early stage disease, in which surgery and trans-arterial chemoembolization (TACE) are the major treatment options. Liao et al. (2016) showed that tumor-associated mutations captured in plasma are more easily detected in patients who suffer vascular invasion. Moreover, patients with detectable ctDNA mutations have a significantly shorter recurrence-free survival (RFS; 89 vs. 365 days) than those with no mutations in plasma liquid biopsies. Interestingly, no significant correlation between detectable ctDNA mutations and the concentration of circulating ccfDNA was found (Liao et al., 2016). Another study (An et al., 2019) indicated that all patients with detectable ctDNA mutations experienced in situ or distant recurrence postoperatively, whereas a small fraction of patients (~30.8 %) without ctDNA somatic mutations experienced recurrence. Moreover, patients with detectable mutations in post-operative plasma had significantly shorter median DFS (8.3 months vs. unreached) than those without detectable mutations (An et al., 2019). Notably, the number of mutations in ctDNA, maximal variant allele frequency (VAF) in ctDNA, and ctDNA concentration linearly correlated with tumor size (An et al., 2019). Furthermore, increased single-nucleotide variant (SNV) and copy-number variant (CNV) fractions detected by targeted deep- and low-coverage WGS were also related to worse clinico-pathological features (i.e., increased tumor size, presence of microvascular invasion, and more severe tumor differentiation) (Cai et al., 2019). Patient groups with high SNV/CNV fractions in pre-operative plasma samples have significantly poorer RFS and OS than patients with low SNV/CNV fractions. Dynamic changes in the SNV and CNV profiles during follow-up correlated with tumor burden and were consistent with imaging results.

The role of the somatic ctDNA profile in predicting the therapeutic outcome was also investigated in the context of patients with advanced HCC treated with systemic treatment (e.g., sorafenib) (Alunni-Fabbroni et al., 2019; Oh et al., 2019). Oh et al. (2019) reported a higher rate of genomic instability when analyzing the genome-wide copy number alterations via low depth WGS, which was significantly associated with lower DCR (52.0 % vs. 75.0 %) and shorter TTP (2.2 vs. 4.1 months) and OS (4.6 vs. 14.8 months) in patients with HCC receiving sorafenib as first-line therapy. Another exploratory investigation (Alunni-Fabbroni et al., 2019) found a significant correlation between ccfDNA levels and the presence of distant metastases and survival when analyzing plasma samples collected before sorafenib-based systemic therapy and during follow-up. Moreover, the genomic profiles of ctDNA evaluated by NGS-based screening of 597 cancer-relevant genes indicated 28 variants in different combinations at the different time points. The hepatocyte nuclear factor 1-alpha (*HNF1A*), Bcl2-associated X protein (*BAX*), and cytochrome P450 2B6 (*CYP2B6*) genes had the highest mutation frequency and a significant association with patients' clinico-pathological characteristics (i.e., portal vein invasion, liver cirrhosis, and BCLC grading) and the survival profile. Taken together, these preliminary findings support the clinical utility of a quantitative and qualitative analysis of circulating DNA to monitor the response to therapy in advanced HCC undergoing systemic treatment.

Despite the potential opportunity to study somatic genetic features by analyzing circulating free tumor DNA, this strategy is hampered by the difficulty of distinguishing between tumor and germline DNA fractions in plasma. Only the detection of somatic mutations in cell-free DNA in plasma can be used to identify the tumor DNA fraction. However, a promising strategy is based on studying the length of circulating tumor DNA fragments in the plasma of HCC patients. Circulating tumor DNA fragments in plasma are shorter (~150 bp) than cell-free DNA derived from healthy tissues and have a different fragmentation pattern. The size of ccfDNA in plasma and the preferred end-coordinates of the DNA fragments have been demonstrated to be related to the tissue of origin, and can be exploited to identify the plasma level

of tumor DNA in HCC patients, aimed at monitoring disease burden during the course of treatment (Chen et al., 2012; Huang et al., 2016; Jahr et al., 2001; Jiang et al., 2015, 2018; Thierry et al., 2016).

5. Immunotherapy with ICIs

5.1. The role of immune checkpoint molecules

The process of T-cell-mediated immunity is defined by an interplay of stimulatory and inhibitory signals capable of promoting adaptive responses against foreign antigens and avoiding autoimmunity. By counteracting active signaling, immune checkpoints exert a key role in central and peripheral tolerance (Xu et al., 2018). Under physiological conditions, immune checkpoint molecules represent a negative feedback to regulate inflammatory responses following T-cell activation (Chambers et al., 2001; Collins et al., 2002; Inarrairaegui et al., 2018; Kalbasi and Ribas, 2020; Krummel and Allison, 1996; Sharma and Allison, 2020; Stone et al., 2009; Wei et al., 2019a, 2018; Wei et al., 2017, 2019b).

During T-cell maturation, following T-cell receptor recognition of specific antigens on the major histocompatibility complexes (MHCs) of antigen-presenting cells, antigen-specific T cells undergo clonal selection, priming, and activation. Thereafter, T cells move to specific sites based on a chemokine gradient. If they encounter cognate antigens on the MHCs of antigen-presenting cells, they promote cytotoxicity. Specifically, in the case of tumor antigens, they can kill tumor cells (Kumar et al., 2018).

Chronic inflammation can render T cells exhausted and upregulate non-redundant inhibitory receptors that limit their effectiveness, including CTLA-4 and PD-1 (Inarrairaegui et al., 2018; Kalbasi and Ribas, 2020; Krummel and Allison, 1996; Sharma and Allison, 2020; Stone et al., 2009; Wei et al., 2019a, 2018; Wei et al., 2017, 2019b). CTLA-4 is a member of the immunoglobulin superfamily homologous to CD28 (Chambers et al., 2001; Collins et al., 2002). However, it has a higher affinity for B7 and can inhibit T-cell activity by competing with CD28 for binding their shared ligands, B7 − 1/2 (CD28/CD86), without inducing subsequent stimulatory signals (Chambers et al., 2001; Collins et al., 2002). CTLA-4 can also induce inhibitory signals capable of counteracting stimulatory signals by CD28/B7 and TCR/MHC binding (Fallarino et al., 1998; Masteller et al., 2000). The intricate balance between CD28/B7 and CTLA-4/B7 defines the activation or anergy state of T cells. CTLA-4 inhibits the T-cell response early in the immune response, mainly in the lymphoid tissues. In naïve T cells, CTLA-4 is located in the intracellular space and is expressed on the cell surface upon stimulatory signals from CD28/B7 and TCR/MHC binding, whereas in Tregs, CTLA-4 is constitutively expressed and involved in the Treg suppressive functions. The B7 ligands for CTLA-4 are expressed by professional antigen-presenting cells, which are principally located in the lymph nodes and spleen (Fife and Bluestone, 2008; Han et al., 2014; Kalbasi and Ribas, 2020; Masteller et al., 2000; Schneider et al., 2006; Wei et al., 2017, 2019a; Wei et al., 2019b; Sharma and Allison, 2020; Wei et al., 2018). In the context of HCC, dendritic cells (DCs) express high levels of CTLA-4 and can suppress the $CD4^+$ T-cell response via IL10 and indoleamine-2,3-dioxygenase (IDO) in a CTLA-4-dependent manner (Han et al., 2014; Kato et al., 2020; Ouyang et al., 2016; Patente et al., 2018; Wang et al., 2011).

PD-1 belongs to the B7/CD28 costimulatory receptor family expressed on DCs, NK cells, activated T cells, B cells, and monocytes (Ishida et al., 1992). The main ligand of PD-1, PD-L1 is expressed on hematopoietic cells, microvascular endothelium cells, and parenchyma cells of different organs, whereas PD-L2, another ligand of PD-1, is expressed on activated macrophages and DCs (Bhandaru and Rotte, 2017; Cheng et al., 2013; Francisco et al., 2010; Hui et al., 2017; Kalbasi and Ribas, 2020; Keir et al., 2008; Rotte et al., 2018; Sharma and Allison, 2020; Wei et al., 2019a, 2018; Wei et al., 2017, 2019b). T-cell survival can be disrupted by the binding of PD-L1/PD-L2 to PD-1, impairing the T-cell response (Butte et al., 2007; Francisco et al., 2009; Latchman, 2001). A marker of T-cell exhaustion is PD-1 expression. PD-1 is expressed by T cells that are highly stimulated or exposed to reduced levels of $CD4^+$ T cells (Wherry, 2011). T-cell proliferation and the production of IL2, interferon gamma (IFN-γ), and tumor necrosis factor alpha (TNF-α) are blocked by PD-1 binding, reducing T-cell survival. When TCR and PD-1 binding occur concomitantly, the signals generated by PD-1 are involved in terminating early TCR signaling and reducing T-cell activation (Keir et al., 2008). In addition, the interaction between PD-L1 and B/-1 can suppress T-cell activity (Butte et al., 2007). Thus, cancer cells expressing PD-L1 and PD-L2 can affect the antitumor immune response (Pardoll, 2012). While CTLA-4 mainly acts in the priming phase of T-cell activation, PD-1 is involved in the effector phase principally in peripheral tissues. Compared to CTLA-4 engagement, PD-1 engagement can interfere with a higher number of T-cell signaling pathways (Kalbasi and Ribas, 2020; Pardoll, 2012; Sharma and Allison, 2020; Wei et al., 2019a, 2018; Wei et al., 2017, 2019b).

PD-1 and PD-L1 expression and the PD-1/PD-L1 interaction have been investigated in the context of HCC in several studies. In particular, they have demonstrated that PD-L1 expression is localized primarily in neoplastic or intra-tumoral inflammatory cells, such as Kupffer cells, compared to neighboring non-tumor liver cells (Wu et al., 2009). Furthermore, the PD-1/PD-L1 interaction contributes to immune suppression in HCC (Wu et al., 2009). With regards to an involvement of the immune response in HCC, $CD8^+$ T cells express high levels of PD-1 with respect to normal tissues. Furthermore, tumors with a high number of TILs exhibit high levels of PD-L1 expression (Semaan et al., 2017; Wu et al., 2009). From a clinical point of view, a dismal prognosis is associated with PD-L1 expression. In particular, shorter OS correlated with high PD-L1 levels (Semaan et al., 2017). A higher risk of HCC relapse or metastasis and cancer-related death are associated with the presence of peri-tumoral hepatocytes positive for PD-L1 expression (Dai et al., 2017).

Several differences exist between the mechanisms of action of CTLA-4 blockade and PD-1/PD-L1 blockade (Parry et al., 2005). The effects of CTLA-4 blockade mainly occur during naïve T-cell activation in lymph nodes by targeting Tregs expressing CTLA-4, inhibiting their activity in removing B7 − 1/2 from the cell surface of antigen-presenting cells (Fife and Bluestone, 2008; Krummel and Allison, 1996). CTLA-4 blockade also occurs at the tumor site by targeting exhausted CTLA-4-expressing T cells and Tregs within the TME. PD-1/PD-L1 blockade mainly affects the effector stage of the immune response by regulating previously activated T cells, especially in peripheral tissues (Fife and Bluestone, 2008; Keir et al., 2008). In the context of the TME, PD-1/PD-L1-expressing tumor cells can deactivate tumor-infiltrating PD-1-expressing T cells (Fife and Bluestone, 2008; Keir et al., 2008).

HCC is recognized as having a considerable degree of immunogenicity because it is capable of expressing a series of tumor-associated antigens (TAAs) or mutation-associated neoantigens (MANAs) derived from specific somatic gene mutations (Cancer Genome Atlas Research Network, 2017; Capurro et al., 2005). The presence of tumor-infiltrating T cells also characterizes HCC, though in several cases, tumor-infiltrating T cells can be irreversibly exhausted or are not specific for TAAs or MANAs (Ringelhan et al., 2018). Furthermore, the presence of several cell types, such as MDSCs, M2 macrophages, and stromal cells, that exert an inhibitory effect through crosstalk involving immune-inhibitory molecules, such as chemokines/cytokines, characterizes the TME of HCC (Ringelhan et al., 2018). The absence of DCs or their inability to induce anti-tumor T-cell clones may be responsible for the lack of an anti-tumor immune response (Ringelhan et al., 2018). The complexity of this system is responsible for the heterogeneous response rates to immunotherapeutic approaches among HCC patients (Ringelhan et al., 2018). Therefore, it is important to determine predictive factors that may affect the response to immunotherapeutic approaches (Onyshchenko, 2018). One of the first proposed biomarkers of the treatment response is PD-L1 expression levels in tumor cells and

myeloid cells, but it has heterogeneous efficacy among the different types of tumors (Onyshchenko, 2018).

A feature that may be associated with tumor sensitivity to ICIs is the presence of $CD8^+$ T-cell infiltration; at least putatively, it is associated with the presence of stimulated T-cell clones versus specific TAAs or MANAs (Onyshchenko, 2018). The tumor mutational load has also been considered to be a predictor of the response to ICIs; in particular, a high mutational load could be associated with a higher probability of having somatic mutations that generate MANAs capable of stimulating specific T-cell clones (Onyshchenko, 2018). A distinctive response to ICI treatment could also be associated with clinical features or levels of specific tissue/serum biomarkers (Onyshchenko, 2018).

5.2. Modalities to overcome resistance to ICIs

5.2.1. Taking advantage of the complementary mechanisms of ICIs

One of the first strategies proposed for overcoming resistance to ICIs is based on the different mechanisms of action of anti-CTLA4 and anti-PD1 antibodies. Anti-CTLA4 plays a major role in T-cell priming, whereas anti-PD1 is mainly involved in later reactivation of effector T-cell responses. Moreover, anti-CTLA4 is involved in the depletion of Tregs in the TME and enhanced antitumor immunity, facilitating broader antigen recognition. Thus, therapeutic strategies based on a combination of anti-CTLA4 and anti-PD1 treatments have been proposed (Cheng et al., 2020; Kalbasi and Ribas, 2020; Wei et al., 2019a; Yau et al., 2019a) (Fig. 3). The concomitant blockade of other checkpoint molecules, such as T-cell immunoglobulin and mucin-domain containing-3 (TIM-3), T cell immunoreceptor with Ig and ITIM domains (TIGIT), and lymphocyte-activation gene 3 (LAG-3), has also been proposed as a strategy to avoid T-cell dysfunction or exhaustion (Hung et al., 2018; Kato et al., 2020; Koyama et al., 2016; Wierz et al., 2018; Woo et al., 2012). However, T-cell activation can also be amplified by the concomitant use of co-stimulatory agonists, such as 41BB, CD40/OX40, inducible T cell co-stimulator (ICOS), and glucocorticoid-induced TNFR-related (GITR) (Hu-Lieskovan and Ribas, 2017; Kato et al., 2020; Lanuza et al., 2019; Mahoney et al., 2015).

5.2.2. Improving the availability of antigens due to cancer cell death by chemotherapeutic drugs

Chemotherapy is generally considered an anticancer drug regimen, mediating the killing of tumor cells by cytotoxicity or permanent arrest of the cell cycle machinery through apoptosis, that is generally an immunologically silent, immunosuppressive, or tolerogenic process, and in some cases ultimately facilitating cancer recurrence. In the past decade, a select class of cytotoxic agents was identified that causes an immunogenic form of apoptosis, termed immunogenic cell death (ICD), a process that alerts the immune system to the presence of dying cells, by stimulating anticancer immunity through the maturation of DCs, activation of CTLs, and induction of the cytotoxic activity of NK cells (Green et al., 2009; Zitvogel et al., 2011).

Well-known ICD inducers are cytostatic agents, such as anthracyclines, oxaliplatin, and bortezomib (Zitvogel et al., 2008). In this chemotherapy-stimulated immune response, a key role is executed by DCs, which are responsible for the engulfment, processing, and presentation of the antigen from dying tumor cells to T lymphocytes. Other changes in the tumor infiltrate, related to the administration of anticancer treatments, are represented by an increased number of T lymphocytes and an increased relative number of CTLs compared to forkhead box P3 (FOXP3)-expressing Tregs. ICD induced by anticancer chemotherapy was first indicated by enhanced clinical responses to conventional cytotoxic chemotherapeutic agents in the presence of these related tumor-specific immune responses (Zitvogel et al., 2011). On the other hand, the presence of severe leukopenia is correlated with a negative response to chemotherapy in solid tumors (Halama et al., 2011). ICD depends on adaptive stress responses promoting the emission of endogenous danger signals from dying cells. These danger signals are transmitted by released or exposed DAMPs. These molecules can be subdivided into proteins exposed on the surface of stressed and dying cells (e.g., calreticulin and heat shock proteins), or a lipid moiety flipping from the inner leaflet of the plasma membrane to the outer leaflet (e.g., phosphatidylserine), proteins or other molecules released into the extracellular milieu by dying cells (e.g., high mobility group box 1 [HMGB1], uric acid, IL1a, pro-inflammatory cytokines), different degradation products, such as ATP, DNA, RNA, and extracellular matrix components such as hyaluronan, heparin sulphate, and degraded matrix constituents (Apetoh et al., 2007; Garg et al., 2012; Ghiringhelli et al., 2009; Krysko et al., 2013). Different factors can influence the immunogenicity of cell death, including the intrinsic antigenicity of cells, an activated cell state, stress prior to cell death, the type of cell death inducer, the cell death pathway involved, and the concomitant presence of immune cells capable of a response (Steinman et al., 2003).

DCs play a key role in DAMP processing. DAMPs released or exposed by stressed, damaged, or dying cells determine the apoptotic engulfment and activation of DCs, antigen processing, DC maturation, and subsequent T-cell activation. The recognition of DAMPs by DCs is carried out by specific receptors terms pattern recognition receptors (PRRs). PRRs expressed by DCs that stimulate antigen uptake are endocytic receptor-like scavengers and C-type lectin receptors. Toll-like receptors (TLRs), nucleotide-binding oligomerization domain (NOD)-like receptors, and retinoic acid-inducible gene I (RIG-I)-like helicases are other PRRs of DCs that stimulate reactions subsequent to antigen uptake (Lim et al., 2011; Steinman et al., 2003).

Conventional chemotherapy relies on the administration of cytotoxic drugs at or close to the maximal tolerated dose (MTD), generally with a 3-week interval. This drug-free period is necessary for the recovery of chemotherapy-induced adverse effects in the patient. However, during the same period, tumor cells may resume growth, particularly through tumor neovascularization via mobilization of circulating endothelial progenitor cells (Shaked et al., 2006). Metronomic chemotherapy based on the more frequent administration of lower doses than those employed for conventional chemotherapy has been demonstrated to reduce toxic side effects and prevent vascular regrowth during therapy breaks (Shaked et al., 2006). Metronomic treatment regimens are generally based on inexpensive, well-tolerated, orally administered drugs, presumably capable of preventing tumor progression for extended periods of time. Although the anti-angiogenic effect was initially considered to be the exclusive antitumor effect obtained by metronomic chemotherapy (Kerbel and Kamen, 2004), other mechanisms of action, including an antitumor immune response, have been associated with this treatment regimen (Pasquier et al., 2010). In particular, induction of ICD has been found to be one of the main immunostimulatory effects exerted by metronomic chemotherapy (Kaneno et al., 2009). Metronomic chemotherapy has also been found to be responsible for enhancement of antigen presentation through DC modulation, increased cancer cell immunogenicity, preferential depletion of regulatory T cells, MDSC modulation, and enhanced cytotoxic activity of tumor effector cells, particularly tumor-specific T cells and γδT cells (Kaneno et al., 2009; Michels et al., 2012; Todaro et al., 2013). As a result of these findings, a combination of metronomic chemotherapy and immunotherapy strategies has been proposed (Fig. 3).

5.2.3. Improving the anti-tumor immune response by radiotherapy

Historically, radiotherapy has been considered a local treatment for cancer with an “in-field” antitumor effect. In particular, radiotherapy has been used to treat localized malignancies with curative intent or, alternatively, to palliate pain, bleeding, or metastasis (Jaffray, 2012). Interestingly, in a proportion of treated patients with multiple lesions, tumor regression has been observed outside the radiotherapy field in non-irradiated metastatic lesions distant from the primary site of irradiation. This phenomenon is called the abscopal effect (Abuodeh et al., 2016; Mole, 1953; Postow et al., 2012). The well-known activity of radiotherapy to which solid tumors respond is radiation-induced DNA

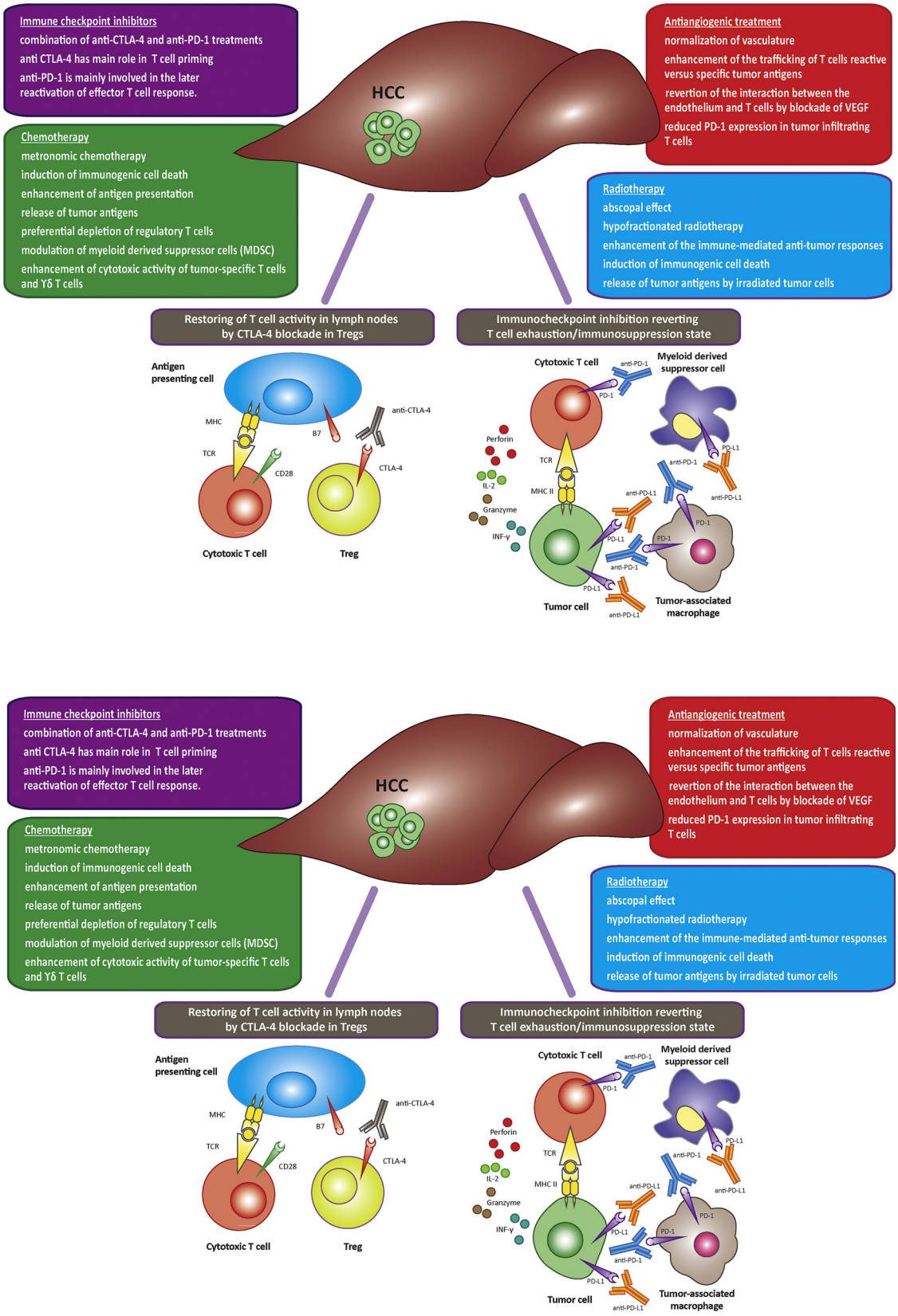


Fig. 3. Interactions of immune checkpoint inhibitors. Cytotoxic T lymphocyte protein 4 (CTLA-4), which is expressed in Tregs, is able to inhibit T-cell activity by competing with CD28 for binding their shared ligands B7-1/2 (CD80/CD86). CTLA-4 blockade acts mainly by targeting CTLA-4-expressing Tregs in the lymph nodes. Programmed cell death protein receptor (PD-1)/programmed death ligand 1 (PD-L1) blockade can overcome the T-cell exhaustion and reverse the immunosuppressive state of the tumor microenvironment by blocking immune checkpoint molecules. The concomitant use of anti-CTLA4 and anti-PD1 antibodies could enhance T-cell effectiveness by acting at both lymph nodes and in the tumor microenvironment. Chemo-immuno- or radio-immuno-treatment combinations could be capable of enhancing immunogenic cell death and the number of tumor neoantigens, and reduce immuno-suppression. The efficacy of immune checkpoint inhibitors could be augmented by concomitant treatment with antiangiogenic molecules to improve the functionality of tumor vessels via the vasculature normalization process.

damage, which causes direct tumor cell death via tumor cell apoptosis, senescence, and autophagy (Dewey et al., 1995; Eriksson and Stigbrand, 2010). This mechanism of action has primarily been considered to be immuno-suppressive because these cytotoxic effects can also affect leukocytes. In patients with solid tumors, leukopenia has been observed following radiotherapy (Campian et al., 2013). Total body irradiation is a well-known conditioning regimen for patients undergoing bone marrow transplantation (Hill-Kayser et al., 2011). On the other hand, activation of the immune system by radiation has been observed, with an increasing role of radiotherapy in the enhancement of immune-mediated anti-tumor responses. The abscopal effect has already been documented in a series of studies in murine models (Demaria et al., 2004; Kingsley, 1975; Reynders et al., 2015). However, the abscopal effect of radiotherapy alone indicates a relatively low overall occurrence rate, though this effect has been reported in a growing number of trials and cases. Nevertheless, given that the host's immune tolerance toward tumors can be reduced by immunotherapy with ICIs, combination regimens of radiotherapy and immunotherapy could amplify the anti-tumor immune response (Demaria et al., 2005; Vatner et al., 2014). This synergistic effect could be due to the capability of radiotherapy to induce ICD and the subsequent release of tumor antigens by irradiated tumor cells, acting as an in situ vaccination. The optimal dose and fractionation of radiotherapy to induce the abscopal effect has not been completely defined (Dewan et al., 2009; Siva et al., 2015). In this context, hypofractionated radiotherapy has been proposed to obtain the systemic anti-tumor effect of combinatorial radiotherapy/immunotherapy regimens (Fig. 3) (Dewan et al., 2009; Twyman-Saint Victor et al., 2015). Another unclear point is represented by the optimal schedule of combinatorial treatments, particularly if the two treatments should be concomitantly or sequentially administered (Dewan et al., 2009; Twyman-Saint Victor et al., 2015). Recently, combining chemotherapeutic cytotoxic agents with ICIs in difficult-to-treat solid tumors, including non-small cell lung cancer and head and neck cancer, was proposed or implemented (Kaidar-Person et al., 2018; Leonetti et al., 2019). The effort to combine current ICIs with novel checkpoint inhibitors or other therapeutic approaches to achieve a synergistic effect in the treatment of various human malignancies is ongoing (Kon and Benhar, 2019).

5.2.4. Reversal of the effect of proangiogenic factors

A number of suggestions have been made concerning the link between the immune system and angiogenesis in the TME. The number and functionality of TILs are reduced by abnormal angiogenesis (Hato et al., 2016). In the context of an immunosuppressive TME, VEGF plays a key role as a mediator in evading immune surveillance by tumor cells. VEGF is associated with the accumulation of MDSCs, which can induce the development of Foxp3-expressing Tregs (Gabrilovich and Nagaraj, 2009). High VEGF levels are also associated with a decrease in DCs capable of achieving a mature state. The presence of immature DCs can be responsible for the differentiation and proliferation of Tregs (Hato et al., 2016). VEGF can also cause the production of TAMs (Mantovani et al., 2017). Angiopoietin-2 (Ang-2) is another pro-angiogenic factor with an immunosuppressive role. In particular, the expression of Ang-2 by tumor cells can cause the recruitment of TIE-2-expressing monocytes responsible for the release of IL10. IL10 production is related to the suppression of T-cell proliferation, augmentation in the CD4+/CD8 + T cell ratio, and the expansion of Foxp3-expressing Tregs (Motz et al., 2014).

The trafficking of T cells reactive towards specific tumor antigens or, alternatively, of other effector cells of the immune system can be induced or increased by anti-angiogenic treatments. In this context, scheduling has been proposed for the administration of anti-angiogenic molecules to achieve a transient therapeutic drug-induced time interval in which tumor vessels demonstrate improved functionality rather than hypoxia, in a process called normalization of the vasculature (Liu et al., 2017). This restored functionality of tumor vessels is potentially useful to augment the capability of additional therapeutic agents, such as ICIs, to eradicate tumor cells if employed in association with the anti-angiogenic molecules (Fig. 3) (Liu et al., 2017).

6. Immunotherapy targeting TAAs

6.1. TAAs expressed in HCC

A number of TAAs specific to HCC have been identified, including Glypican-3 (GPC3) (Anatelli et al., 2008; Capurro et al., 2003; Zhang et al., 2012), epithelial cell adhesion molecule (EpCAM) (Ogawa et al., 2014; Yamashita et al., 2008), and tumor endothelial marker 1 (TEM1) (Bagley et al., 2008; Christian et al., 2008). GPC3 belongs to the glypican family (Filmus et al., 2008; Filmus and Selleck, 2001) and is an oncofetal protein expressed in the embryo during development that is involved in morphogenesis and growth control. In contrast, GPC3 is not expressed in most adult healthy tissues (Capurro et al., 2008). With respect to normal liver and pre-malignant lesions, GPC3 expression at both the transcript and protein levels is upregulated in HCC (Anatelli et al., 2008; Capurro et al., 2003; Yamauchi et al., 2005; Zhang et al., 2012). Immunohistochemistry has shown strong positivity for both membrane and cytoplasmic staining in a large proportion of HCC cases (Capurro et al., 2003; Liu et al., 2010). Furthermore, the concentration of soluble GPC3 is high in the serum of HCC patients, whereas very low or undetectable levels have been found in the serum of healthy individuals or of patients with hepatitis (Capurro et al., 2003; Liu et al., 2010). Therefore, GPC3 can be a promising target for immunotherapy (Capurro et al., 2003, 2008; Liu et al., 2010).

EpCAM is a type I transmembrane glycoprotein that plays a role in cell adhesion, cell migration, cell differentiation, proliferation, metastasis, cell signaling, cellular metabolism, regeneration, and liver organogenesis. EpCAM has frequently been found to be overexpressed in epithelial carcinomas, including hepatic carcinomas (Ogawa et al., 2014; Yamashita et al., 2008).

TEM1 (CD248, also known as endosialin) is a sialic acid-rich transmembrane glycoprotein of the C-type, lectin-receptor family that is expressed on the cell surface of mesenchymal stem cells, endothelial progenitor cells, and fibroblasts during embryonic development (Bagley et al., 2008; Christian et al., 2008). TEM1 expression has been found in several cancers, including HCC, at both the transcript and protein levels (Nanda et al., 2006). TEM1 is also involved in the vascular adhesion and migration of tumor cells, local invasion, and metastasis (Nanda et al., 2006).

6.2. Treatment strategies using antibodies targeting TAAs

Several treatment strategies using antibodies targeting TAA have been proposed for the treatment of HCC. In particular, condrituzumab (GC33) is a fully humanized recombinant IgG2 mAb that is directed to the C-terminal region of human GPC3 (Ishiguro et al., 2008). Condrituzumab is capable of inducing antibody-dependent cell-mediated cytotoxicity (ADCC) and inhibits cancer growth in a xenograft model of liver tumors (Ishiguro et al., 2008). Several clinical trials have been proposed for the use of condrituzumab in HCC: a phase I clinical trial in which it was well-tolerated in patients with advanced HCC (NCT00746317) (Zhu et al., 2013); a randomized phase II clinical trial (NCT01507168) in which it did not show clinical benefits in previously treated HCC patients (Abou-Alfa et al., 2016); and a third trial in which condrituzumab was administered in combination with sorafenib, but did not elicit strong anti-tumor activity (NCT00976170) (Abou-Alfa et al., 2017). Another monoclonal antibody targeting GPC3 is YP7 (Phung et al., 2012; Zhang and Ho, 2016), a humanized IgG1 mAb against the C-terminal region of GPC3. This antibody is capable of inducing ADCC and complement-dependent cytotoxicity in tumor cells expressing GPC3 (Zhang and Ho, 2016). YP7 is also capable of inhibiting tumor growth in nude mice with HCC xenografts (Phung et al.,

2012). A third antibody capable of targeting GPC3 is NH3, a human heavy chain variable domain antibody that targets a unique conformational epitope in the GPC3 core (Feng et al., 2013). Tumor cell growth in a nude mouse HCC xenograft model has been shown to be inhibited by the NH3 antibody, and this inhibition appeared to be associated with inactivation of YAP (Feng et al., 2013), an established transcriptional coactivator promoting tumor cell proliferation in HCC (Zhang and Zhou, 2019). Ontuxizumab is a humanized IgG1/K mAb targeting TEM1. In a first-in-human phase I clinical trial (NCT00847054) of patients affected by different treatment refractory solid tumors, HCC patients achieved disease stabilization (Diaz et al., 2015). In a second phase I trial (NCT01773434) of a group of patients affected by various solid tumors, the HCC patients presented with tumor shrinkage (Doi et al., 2019).

6.3. CAR-T

Adoptive T-cell transfer consists of the isolation of T cells from patient blood and reinfusion of the most antigen-specific T cells after their stimulation and expansion. This approach became a therapeutic strategy using CAR-T cells, which are engineered to recognize a specific TAA. Four components characterize CAR-T, the first, and external, component is usually made by the single− chain variable fragment (scFv) domain. The second component, the space region or hinge, consists of the IgG1 hinge−CH2−CH3 Fc domain connecting the scFv. The third component consists of the transmembrane region. The last component is the intracellular signaling domain, the composition of which differs from one CAR-T generation to another, with the permanent presence of the CD3ζ signaling domain in all of them.

Progress in the development of the CAR could be roughly defined by the fifth generation CAR-T. The first generation is the simplest, with CD3ζ as the intracellular signaling domain. The second generation is characterized by the addition of a costimulatory domain, CD28 or 4−1BB, between the transmembrane domain and CD3ζ to determine T-cell proliferation and expansion following repeated exposure of T cells to the antigen. The third generation CAR-T has both CD28 and 4−1BB (or CD134) co-stimulatory domains bound to the CD3ζ. The fourth generation CAR-T comprises T cells redirected for universal cytokine killing (TRUCKs) and has a costimulatory domain and inducible IL12 (iIL12) cassette. In this case, the interaction between the CAR and the antigen activates T-cell signaling, resulting in the release of the pro-inflammatory IL12, which allows the recruitment of NK cells and macrophages able to attack even the antigen-negative cells. A fifth generation of CAR-T is characterized by the presence of a truncated cytoplasmic IL2 receptor β-chain domain with a binding site for the transcription factor signal transducer and activator of transcription 3 (STAT3). In this way, synergistic signals for T-cell activation and proliferation are generated by the antigen-specific activation that simultaneously triggers the TCR (through the CD3ζ domain), co-stimulatory domains (CD28 domain), and cytokine Janus kinase (JAK)-STAT3/5 signaling (Kagoya et al., 2018) (Fig. 4).

6.3.1. Approved CAR-T for the treatment of hematological malignancies

CAR-T therapy was approved by the FDA in 2017 for the agents tisagenlecleucel (Kymriah) and axicabtagene ciloleucel (Yescarta). Tisagenlecleucel is made of an anti-CD19 scFv with a transmembrane domain bound to 4−1BB and CD3ζ. This therapy has been approved for the treatment of patients aged ≤ 25 years with second or third relapse or refractory B cell acute lymphoblastic leukemia (B-ALL). Axicabtagene ciloleucel is made of an anti-CD19 scFv with a transmembrane domain bound to CD28 and CD3ζ. The drug has been approved for the treatment of adults encountering a relapse or treatment-resistant diffuse large B cell lymphoma (DLBCL) after two or more lines of systemic therapy (Maude et al., 2018; Neelapu et al., 2017).

6.3.2. CAR-T approaches for HCC treatment

CAR-T treatment in solid tumors is favored by the positive results of CAR-T therapy in hematological malignancies. The treatment of HCC or HCC metastasis using CAR-T therapy against different TAAs has been the subject of several studies (Burga et al., 2015; Gao et al., 2014; Jiang et al., 2016; Katz et al., 2015; Zhang et al., 2016). in vivo studies have demonstrated the efficacy of anti-GPC3 CAR-T therapy when treating a mouse model harboring HCC xenografts obtained from patient-derived xenografts or cell line based-tumors (Gao et al., 2014; Jiang et al., 2016). Currently, 22 clinical trials are investigating the use of CAR-T for HCC treatment, 11 of which are using GPC3 as the target (Table 1).

6.3.3. Challenges of CAR-T therapy in solid tumors

The clinical efficacy of CAR-T therapy in hematological malignancies paved the way for the use of CAR-T treatment in solid tumors. However, different from hematological tumors, the treatment of solid tumors presents several challenges, including tumor heterogeneity in terms of antigen expression, access to the tumor site by CAR-T cells, and resistance of the TME to CAR-T therapy.

Most solid tumors are characterized by a heterogeneous cell population due to mutational events, only a subset of tumor cells expressing the specific TAA chosen as a target, or tumor cells having lost expression of the target antigen, making it difficult to target a specific TAA. To overcome both antigen heterogeneity and antigen loss, one approach is to simultaneously target more than one TAA with multi-specific CAR-T cells (Zah et al., 2016). An ideal TAA target must be expressed on the surface of tumor cells. Approximately 1% of total cellular proteins are actually expressed on the cell surface; therefore, only a few TAAs could potentially be used as a target for CAR-T therapy (Walseng et al., 2017).

TAAs are commonly enriched in tumors, but also expressed at low levels in normal tissues. This could potentially lead to toxicity due to on-target/off-tumor recognition (Morgan et al., 2010; Richman et al., 2018). Thus, identifying a safe TAA is important. A CAR-T cell must reach the tumor site, but solid tumors have multiple barriers that a CAR-T cell must surmount. Physical barriers, such as cancer associated fibroblasts and abnormal vasculature at the tumor site, can block T-cell entry (Hanahan and Coussens, 2012; Vignali and Kallikourdis, 2017). In addition, surface markers, such as selectins on endothelial cells, can bind the circulating CAR-T cells, reducing their availability at the tumor site.

Limited CAR-T-cell accessibility to the tumor is overcome by direct intra-tumoral delivery of T cells. For tumors that are not easily accessible, CAR-T cells have been modified to express extracellular matrix-degrading enzymes. Several studies have shown the capability of tumor cells or tumor-associated stromal cells to produce chemokines promoting cell proliferation, survival, progression and migration (Chow and Luster, 2014; Hanahan and Weinberg, 2011). The recruitment of immuno-suppressive cells that further enhances the immunosuppressive TME can be promoted by some of these chemokines. A proposed approach is to take advantage of this tumor chemokine signaling network to drive CAR-T cell recruitment by engineering the expression of cognate chemokine receptors on the CAR-T-cell surface to potentiate their infiltrative capacity (Hillerdal and Essand, 2015; Rapp et al., 2016).

TME has been reported to be a hostile environment for CAR-T cells in regards to survival and proliferation. The glycolytic metabolism of tumor cells renders the environment hypoxic, acidic, low in nutrients, and prone to oxidative stress. Because glucose is depleted by tumor cells, the glycolytic T cells can be nutrient-deprived, causing suppression of the immune response. One approach designed to protect T cells from oxidative stress is CAR-T cells designed to co-express catalase (CAR-CAT), an enzyme that reduces hydrogen peroxide to water and oxygen (Ligtenberg et al., 2016).

In vitro data indicate that CAR-CAT has a reduced oxidative state and improved cellular proliferation compared to CAR alone. These physical and metabolic barriers prevent immune cell recruitment and

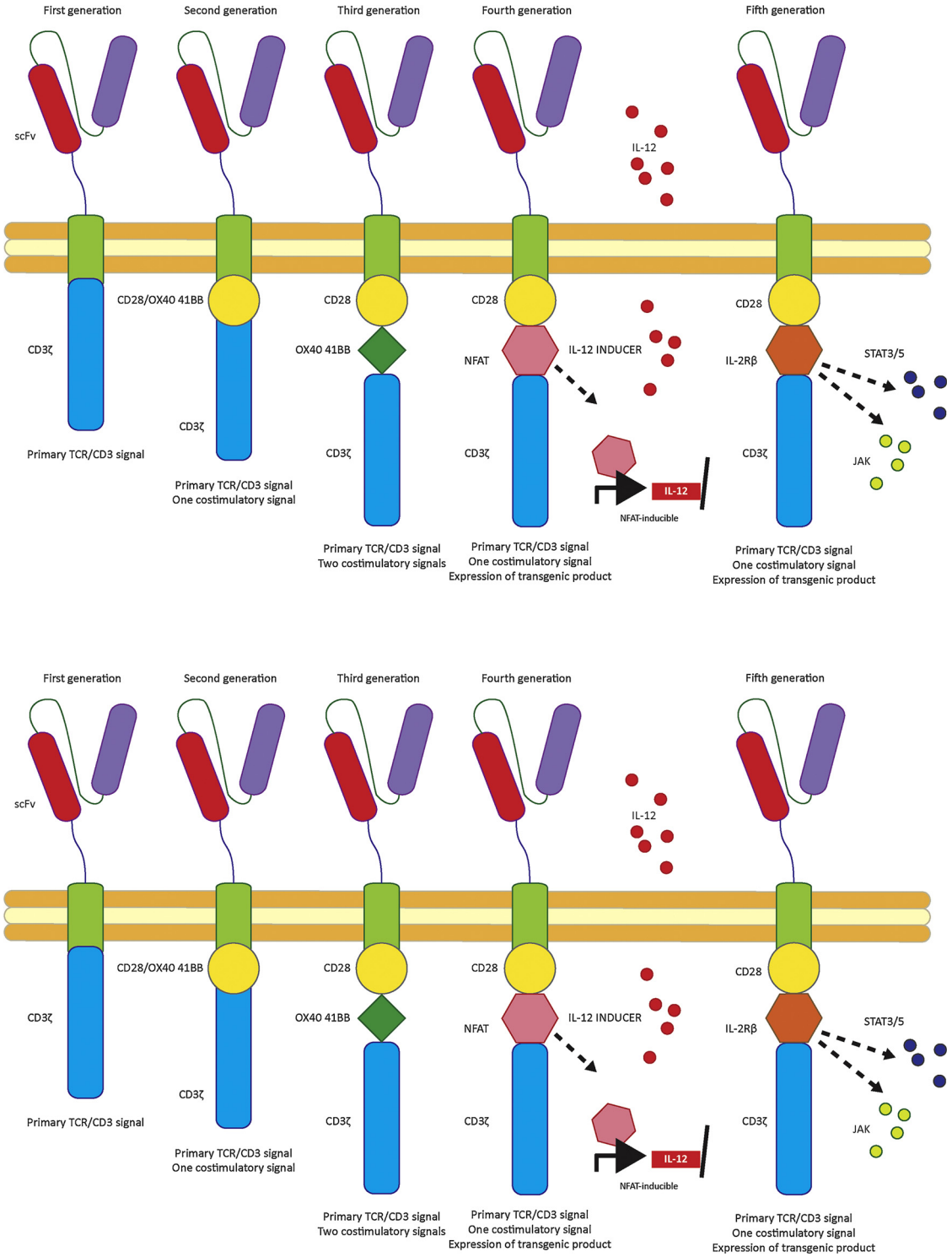


Fig. 4. Chimeric antigen receptor (CAR) structure. Schematic representation of the CAR structure. The first generation CAR contains a CD3ζ-derived signaling domain. The second generation CAR also contains a costimulatory domain (CD28, OX40, or 4-1BB domain), the third generation contains two costimulatory domains, whereas the fourth generation contains a costimulatory domain and inducible interleukin 12 (iIL12) cassette. The fourth generation CAR-T cells are T cells redirected for universal cytokine killing (TRUCKs). In this case, the interaction between the CAR and the antigen activates T-cell signaling, resulting in the release of pro-inflammatory IL12 which allows the recruitment of NK cells and macrophages, which are able to attack even the antigen-negative cells. Fifth generation CAR is characterized by the presence of a truncated cytoplasmic IL2 receptor β-chain domain with a binding site for the signal transducer and activator of transcription 3 (STAT3). In this way, synergistic signals for T-cell activation and proliferation are generated by the antigen-specific activation that simultaneously triggers the TCR through the CD3ζ domain, and costimulatory (CD28 domain) and cytokine Janus kinase (JAK)-STAT3/5 signaling.

Table 1
Clinical trials in HCC using CAR-T.

Title	Condition or disease	Intervention/treatment	NCT number	Status	Phase
A Study of CD147-targeted CAR-T by Hepatic Artery Infusions for Very Advanced Hepatocellular Carcinoma	Advanced Hepatocellular Carcinoma	Biological: CD147-CART	NCT03993743	Recruiting	I
GPC3-targeted CAR-T Cell for Treating GPC3 Positive Advanced HCC	Hepatocellular Carcinoma	Biological: CAR-T cell immunotherapy	NCT04121273	Recruiting	I
Anti-GPC3 CAR-T for Treating GPC3-positive Advanced Hepatocellular Carcinoma (HCC)	Hepatocellular Carcinoma	Biological: Retroviral vector-transduced autologous T cells to express anti-GPC3 CARs Drug: Fludarabine Drug: Cyclophosphamide	NCT03084380	Not yet recruiting	I/II
Clinical Study on the Efficacy and Safety of c-Met/PD-L1 CAR-T Cell Injection in the Treatment of HCC	Primary Hepatocellular Carcinoma	Biological: c-Met/PD-L1 CAR-T cell injection	NCT03672305	Not yet recruiting	Early Phase I
CAR-T Cell Immunotherapy for HCC Targeting GPC3	GPC3 Positive Hepatocellular Carcinoma	Biological: CAR-T cell immunotherapy	NCT02723942	Completed	I/II
Clinical Study of ET1402L1-CAR T Cells in AFP Expressing Hepatocellular Carcinoma	Hepatocellular Carcinoma Liver Cancer Liver Neoplasms Metastatic Liver Cancer	Biological: autologous ET1402L1-CART cells	NCT03349255	Terminated	I
Anti-GPC3 CAR T for Treating Patients With Advanced HCC	Hepatocellular Carcinoma	Biological: anti-GPC3 CAR T	NCT02395250	Completed (GPC3 CAR-T showed a safety profile in Chinese patients with refractory or relapsed GPC3 + HCC holding antitumor activity in combination with lymphodepleting conditioning)	I
Glypican 3-specific Chimeric Antigen Receptor Expressing T Cells for Hepatocellular Carcinoma (GLYCAR)	Hepatocellular Carcinoma	Genetic: GLYCAR T cells Drug: Cytoxan Drug: Fludarabine	NCT02905188	Recruiting	I
T Cells co- Expressing a Second Generation Glypican 3-specific Chimeric Antigen Receptor With Cytokines Interleukin-21 and 15 as Immunotherapy for Patients With Liver Cancer (TEGAR)	Hepatocellular Carcinoma Hepatoblastoma	Genetic: TEGAR T cells Drug: Cytoxan Drug: Fludarabine	NCT04093648	Not yet recruiting	I
GPC3-T2-CAR-T Cells for Immunotherapy of Cancer With GPC3 Expression	Hepatocellular Carcinoma Squamous Cell Lung Cancer	Biological: GPC3 targeting CAR-T cells	NCT03198546	Recruiting	I
4th Generation Chimeric Antigen Receptor T Cells Targeting Glypican-3	Advanced Hepatocellular Carcinoma	Drug: CAR-GPC3 T Cells	NCT03980288	Recruiting	I
A Study of GPC3-targeted T Cells by Intratumor Injection for Advanced HCC (GPC3-CART)	Hepatocellular Carcinoma	Drug: GPC3-CART cells	NCT03130712	Unknown	I/II
CAR-GPC3 T Cells in Patients With Refractory HCC	Hepatocellular Carcinoma	Genetic: CAR-GPC3 T cells	NCT03146234	Completed	Not applicable
A Study of GPC3 Redirected Autologous T Cells for Advanced HCC	Hepatocellular Carcinoma	Drug: TAI-GPC3-CART cells	NCT02715362	Unknown	I/II
Chimeric Antigen Receptor T Cells Targeting Glypican-3	Hepatocellular Carcinoma	Biological: CAR-GPC3 T Cells	NCT03884751	Recruiting	I
NKG2D-based CAR T-cells Immunotherapy for Patient With r/r NKG2DL + Solid Tumors	Hepatocellular Carcinoma Glioblastoma Medulloblastoma Colon Cancer	Biological: NKG2D-based CAR T-cells	NCT0427046	Not yet recruiting	I
Phase I/II Study of Anti-Mucin1 (MUC1) CAR T Cells for Patients With MUC1 + Advanced Refractory Solid Tumor	Hepatocellular Carcinoma Non-small Cell Lung Cancer Pancreatic Carcinoma Triple-Negative Invasive Breast Carcinoma	Biological: anti-MUC1 CAR T Cells	NCT02587689	Unknown	I/II
Autologous CAR-T/TCR-T Cell Immunotherapy for Solid Malignancies	Esophagus Cancer Hepatoma Glioma Gastric Cancer	Biological: CAR-T/TCR-T cells immunotherapy	NCT03941626	Recruiting	I/II

(*continued on next page*)

Table 1 (*continued*)

Title	Condition or disease	Intervention/treatment	NCT number	Status	Phase
Clinical Study of Redirected Autologous T Cells With a Chimeric Antigen Receptor in Patients With Malignant Tumors	B Cell Lymphoma B Cell Leukemia Myeloma Hepatocellular Carcinoma Pancreatic Carcinoma Adenocarcinoma of Esophagogastric Junction	Genetic: CAR-CD19 T cell Genetic: CAR-BCMA T cell Genetic: CAR-GPC3 T cell Genetic: CAR-CLD18 T cell Drug: Fludarabine Drug: Cyclophosphamide	NCT03302403	Recruiting	Not applicable
A Clinical Research of CAR T Cells Targeting EpCAM Positive Cancer	Colon Cancer Esophageal Carcinoma Pancreatic Cancer Prostate Cancer Gastric Cancer Hepatic Carcinoma	Biological: CAR-T cell immunotherapy	NCT03013712	Recruiting	I/II
Autologous CAR-T/TCR-T Cell Immunotherapy for Malignancies	B-cell Acute Lymphoblastic Leukemia Lymphoma Myeloid Leukemia Multiple Myeloma Hepatoma Gastric Cancer Pancreatic Cancer Mesothelioma Colorectal Cancer Esophagus Cancer Lung Cancer Glioma Melanoma Synovial Sarcoma Ovarian Cancer Renal Carcinoma	Biological: CAR-T cell immunotherapy	NCT03638206	Recruiting	I/II
A Study of Chimeric Antigen Receptor T Cells Combined With Interventional Therapy in Advanced Liver Malignancy	Hepatocellular Carcinoma Pancreatic Cancer Metastatic Colorectal Cancer Metastatic	Drug: CAR-T cell	NCT02959151	Unknown	I/II

Shown are the clinical trials reported on ClinicalTrials.gov by searching the keywords: “CAR-T” versus “hepatocellular carcinoma” (search performed on 2020, March 19th. Abbreviations: NCT number: ClinicalTrials.gov identifier.

the activation and persistence of CAR-T cells, and simultaneously promote the recruitment of immune suppressor cells (Hanahan and Weinberg, 2011; Wu et al., 2015). These immune suppressor cells are involved in CAR-T-cell exhaustion, with the sequential loss of effector functions, such as the ability to produce cytokines, proliferate, and mediate the lysis of target cells. One of the most popular strategies to overcoming T-cell exhaustion is the use of ICIs. Drugs targeting PD-L1, PD-1, and CTLA-4 used in combination with CAR-T therapy have been found to be effective (Chong et al., 2017; Gargett et al., 2016; Heczey et al., 2017).

Other novel approaches are represented by knocking out genes encoding T-cell inhibitory receptors or signaling molecules, such as PD-1 or CTLA-4, particularly via CRISPR-CAS9 technology (Baylis and McLeod, 2017; Ren et al., 2017; Rupp et al., 2017). The responsiveness of CAR-T cells could be prolonged by activating chimeric switch receptors (CSRs), which are also defined as immunomodulatory fusion proteins. These activating CSRs combine the extracellular ligand-binding domain of an inhibitory receptor (PD-1 or CTLA-4) fused through a transmembrane domain with the cytoplasmic co-stimulatory signaling domain of CD28 (Ankri et al., 2013; Kobold et al., 2015; Liu et al., 2016; Prosser et al., 2012; Shin et al., 2012). In this manner, engagement of the extracellular portion of this fusion receptor transmits an activating signal instead of the normal physiological inhibitory signal (Ankri et al., 2013; Kobold et al., 2015; Liu et al., 2016; Prosser et al., 2012; Shin et al., 2012). An alternative strategy for overcoming tumor-mediated immune suppression involves the use of TRUCKs, which express in a constitutive or inducible manner, pro-inflammatory cytokines, such as IL12, that strongly enhance the response of the innate and adoptive immunity against cancer cells (Trinchieri, 2003). In particular, IL12 is capable of increasing IFN-γ secretion and the expression of granzyme B and perforin by T cells and NK cells, and of suppressing the proliferation of Tregs (Cao et al., 2009; Ferlazzo et al., 2004; Kubin et al., 1994).

7. Future perspectives

Despite recent advances in the available therapeutic options, HCC continues to be an incurable disease. Inter-individual variability in terms of tumor response and the emergence of drug resistance remain the main obstacles.

Genomic and transcriptomic heterogeneity is considered one of the principal causes of treatment failure, as well as the lack of selective molecular targets for drug therapy. For the future it could be crucial to start from studying genomic instability of HCC tumors in order to discover new effective treatment strategies and to overcome drug resistance. To achieve this goal, a better understanding of the disease etiology, the functional analysis of genomic instability and the analysis of the mutational burden of HCC, will be of paramount importance. The definition of these aspects of the disease will help not only to discover new selective molecular biomarkers, but to also define new therapeutic strategies in HCC.

HCC is considered a highly immunogenic tumor which is presumably related to its pathogenesis, originating from a chronically inflamed microenvironment of an underlying liver disease, caused by viral or non-viral pathogenic processes. Moreover, it was recently highlighted that the somatic mutational burden, may trigger the immunological response against cancer (Onyshchenko, 2018; Ringelhan et al., 2018). The opportunity to use immunological strategies in HCC patients appears attractive. Anti PD1/PDL1 agents have been recently approved for HCC treatment, albeit the clinical impact of using nivolumab and pembrolizumab on patients' OS of HCC is limited. The success of immunological therapies could be hampered by several factors probably related to the heterogeneity and multifocal origin of HCC. This requires a multimodality strategy combining different immunological approaches (i.e. of anti-CTLA-4 and anti-PD1 antibodies) combined with conventional therapy (i.e. chemotherapy and radiotherapy). Recent evidence suggests an immunological stimulation exerted by the administration of some chemotherapeutics including anthracyclines, that have proved to have some activity in HCC (Apetoh et al., 2007; Garg et al., 2012; Ghiringhelli et al., 2009; Krysko et al., 2013; Zitvogel et al., 2008, 2011). This finding may represent a rational support to the use of chemo-immunotherapy combinations in HCC.

The immunogenic effect of radiotherapy, as evidenced by the so called abscopal effect of radiotherapy (Abuodeh et al., 2016; Demaria et al., 2004; Kingsley, 1975; Mole, 1953; Postow et al., 2012; Reynders et al., 2015), could also redefine the role of radiotherapy in this malignancy, commonly considered not radiosensitive. In the era of immunotherapy, radiotherapy could become a suitable strategy for HCC in the context of combination with immunotherapies. The effect of physical treatments (i.e. radiofrequency or thermoablation) on tumor immunogenicity is not currently clear but it could be hypothesized to be a further therapeutic tool to be investigated as an immunostimulatory agent (Abuodeh et al., 2016; Demaria et al., 2004; Kingsley, 1975; Mole, 1953; Postow et al., 2012; Reynders et al., 2015).

A pharmacogenetic approach is mandatory to better address the issue of drug resistance in HCC. Germline polymorphisms in genes encoding for proteins involved in the drug targeted pathways as angiogenesis have been investigated for patients treated with first line sorafenib, (Gong et al., 2017; Keating, 2017; Vasilyeva et al., 2015). These studies should be extended not only to the new drugs approved by the regulatory agencies for HCC, but also to better define the immunogenetic profile of HCC patients. This could better define the heterogeneous response to immunotherapeutic treatments in HCC patients.

The existence of predictive and prognostic biomarkers that may be used to monitor the outcome of a pharmacological treatment in HCC remains an open question. In this context, the molecular characterization of tumor cells will be the main path to reach validated monitoring tools. A comprehensive multi-gene somatic analysis integrated with immunogenic profiling approaches seem to be the most promising strategy to extrapolate drug-sensitivity scores. Application of a liquid biopsy approach to mine somatic genomic features from circulating tumor DNA in plasma will be helpful to monitor HCC patients during the course of treatment.

Finally, a future attractive perspective for the HCC treatment derives from the use of cell therapies in solid tumors and in particular by using CAR-T cells. HCC is considered a suitable tumor for this strategy due not only to the lack of available effective treatments but also because it represents a suitable strategy in a tumor with complex immunological features.

8. Conclusions

Sorafenib is the most common first-line systemic treatment for HCC, but other targeted therapies with small molecules or immunotherapy with ICIs have been used recently. Despite promising results, HCC remains a drug-resistant tumor. Pharmacogenetics has been applied in the last few years in the personalization of systemic treatments for HCC patients. Currently, all published studies aiming to discover potential genetic predictors of the host drug response have been focused on sorafenib, which has the longest track record in HCC treatment. The most consistent data were obtained for polymorphisms in the VEGF/VEGFR cascade (i.e., VEGFA and VEFGR2) and directly related pathways (*eNOS, HIF1A,* and *ANG2*). However, larger independent prospective trials are required to validate the real clinical utility in stratifying patients according to sorafenib responsiveness. Regarding the other drugs approved for HCC treatment, no pharmacogenetic data have yet been obtained in the specific setting of HCC, and future studies are certainly warranted. Given the recent extension of therapeutic options for HCC treatment, the identification of genetic predictors of good responders could certainly contribute, in combination with other biomarkers, to improving the application of these new drugs in personalized treatments for HCC.

Genomic and transcriptomic heterogeneity is considered one of the principal causes of treatment failure, especially for sorafenib. Considering the complex molecular genomic profile and cancer biology of HCC, which is the end-product of chronic liver injury of various etiologies, a comprehensive understanding of the molecular alterations found in individual cancers is far from being reached; novel methods or platforms that could eventually integrate and interpret these complex data sets and accurately predict response rates to specific targeted therapies are needed (Sullivan et al., 2018). Moreover, the identification of targetable genetic alterations could also offer patients the opportunity to be treated with experimental therapies or off-label targeted agents. The quantitative and qualitative evaluation of ccfDNA as a potential surrogate for tumor molecular profiling is a promising strategy for improving the management of HCC patients. In particular, quantitative alterations in ccfDNA could represent a tool to dynamically monitor the efficacy of treatment in late-stage HCC treated with systemic therapy. Furthermore, a qualitative ctDNA analysis (i.e., ctDNA mutational profile and tumor burden) could permit the identification of mechanisms of resistance to targeted agents (i.e., TKIs and ICIs) or used to guide tumor-targeted therapy and personalized medicine.

HCC is considered to have a significant degree of immunogenicity. The liver plays a central role in host defense and the maintenance of immune tolerance, and is characterized by a strong intrinsic immunosuppressive microenvironment and high immune invasion. This intricate balance between immunostimulatory and immunosuppressive factors in the context of the TME of HCC, results in heterogeneous response rates to ICIs. Though a fraction of HCC cases are responsive to ICIs, the non-responsive cases could benefit from therapeutic strategies capable of augmenting this responsiveness. In this regard, the concomitant use of anti-CTLA-4 and anti-PD1 antibodies could enhance T-cell effectiveness by acting at both the lymph nodes and TME. Chemo-immuno- or radio-immuno-treatment combinations could be capable of enhancing ICD and the number of tumor neoantigens, and alleviating immune suppression. The efficacy of ICIs could be augmented by concomitant treatment with antiangiogenic molecules to improve the functionality of tumor vessels via vasculature normalization (Fig. 3).

Various genomic, transcriptomic, and immune-related features can be useful for identifying patients for whom the better choice is ICI immunotherapy as a single agent or combination therapies, including the somatic features of tumor cells, mutational landscapes, mismatch-repair deficiency, the number of germline polymorphisms in regulatory genes of the immune system, transcription factors, immune-related miRNA signatures, and exposure to environmental antigens that can possibly influence the T-cell repertoire (Gajewski et al., 2013; Ringelhan et al., 2018). However, temporal intra-patient and/or spatial dynamic variations in the chosen predictive biomarkers are also possible due to intra-tumor heterogeneity. An approach using computational/mathematical models could allow a better understanding of this molecular complexity.

Pre-clinical and clinical evidence has highlighted the potential use of CAR-T cells to treat HCC patients (Burga et al., 2015; Gao et al., 2014; Jiang et al., 2016; Katz et al., 2015; Zhang et al., 2016). Although CAR-T therapy has been successfully evaluated and recently approved for the treatment of hematological malignancies, the efficacy of the CAR-T approach for the treatment of solid tumors, including HCC, is more dependent on an efficient homing of CAR-T cells to the tumor site, and on the capability of CAR-T cells to survive in the TME. An efficient CAR-T strategy for the treatment of HCC could be obtained by selecting specific T-cell subtypes to be engineered, and by the introduction of additional modifications to overcome the immunosuppressive TME, such as the production of cytokines (e.g., IL2) and/or the manipulation of immune checkpoint signaling in which CAR-T cells could be involved (Pang et al., 2018).

Ever-increasing knowledge of the molecular mechanisms involved in HCC pathogenesis and progression, as well as in the tumor/TME/immune system crosstalk, will hopefully allow the rapid introduction of novel effective therapeutic strategies combining anti-neoplastic and immunotherapeutic approaches for the treatment of HCC.

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