

Cellular and molecular targets for the immunotherapy of hepatocellular carcinoma

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Abstract Liver cancer is the sixth most common cancer worldwide and 3rd most common cause of cancer-related death. Hepatocellular carcinoma (HCC) represents more than 90% of primary liver cancer and is a major public health problem. Due to the advanced stages of HCC at the time of diagnosis, utilizing the conventional treatment for solid tumors frequently ends with treatment failure, recurrence, or poor survival. HCC is highly refractory to chemotherapy and other systemic treatments, and locoregional therapies or selective internal radiation therapies are largely palliative. Considering how the pathogenesis of HCC often induces an immunosuppressed state which is further amplified by post-treatment recurrence and reactivation, immunostimulation provides a potential novel approach for the treatment of HCC. Immune response(s) of the body may be potentiated by immunomodulation of various effector cells such as B-cells, T-cells, Treg cells, natural killer cells, dendritic cells, cytotoxic T-lymphocytes, and other antigen-presenting cells; cellular components such as genes and microRNA; and molecules such as proteins, proteoglycans, surface receptors, chemokines, and cytokines. Targeting these effectors individually has helped in the development of newer therapeutic approaches; however, combinational therapies targeting multi-faceted

biomarkers have yielded better results. Still, there is a need for further research to develop novel therapeutic strategies which may act as either complementary or an alternative treatment to the standard therapy protocols of HCC. This review focuses on potential cellular and molecular targets, as well as the role of virotherapy and combinational therapy in the treatment of HCC.

Keywords Hepatocellular carcinoma · Immunotherapy · Immunostimulation · Cellular targets · Molecular targets · Virotherapy · Combinational therapy

Introduction

Liver cancer is the sixth most common cancer diagnosis (749,000 new cases) worldwide and 3rd most common cause of cancer-related death. Hepatocellular carcinoma (HCC) represents more than 90% of primary liver cancer and is a major public health problem [1]. Chronic infection with hepatitis B virus (HBV) or hepatitis C virus (HCV) causes an estimated 75% of all HCC cases. The incidence of HCC due to HCV infection is predicted to increase in western countries until the 2020s [2, 3]. Chronic hepatitis B virus infection, triggering 80% of all liver cancer, is a major cause of chronic liver disease, cirrhosis, and hepatocellular carcinoma [4] (Fig. 1). Nearly 1.4 million deaths occur annually due to chronic liver disease [5], and HCC is the foremost cause of death in cirrhotic patients [6]. Early diagnosis of HCC has a good prognosis with a 5-year survival of more than 70% while late diagnosis has a poor prognosis with a 5-year survival rate of less than 16% [1].

In a healthy liver, inflammation stimulates the process of growth and repair and restores the normal liver architecture. However, with chronic inflammation, the imbalance

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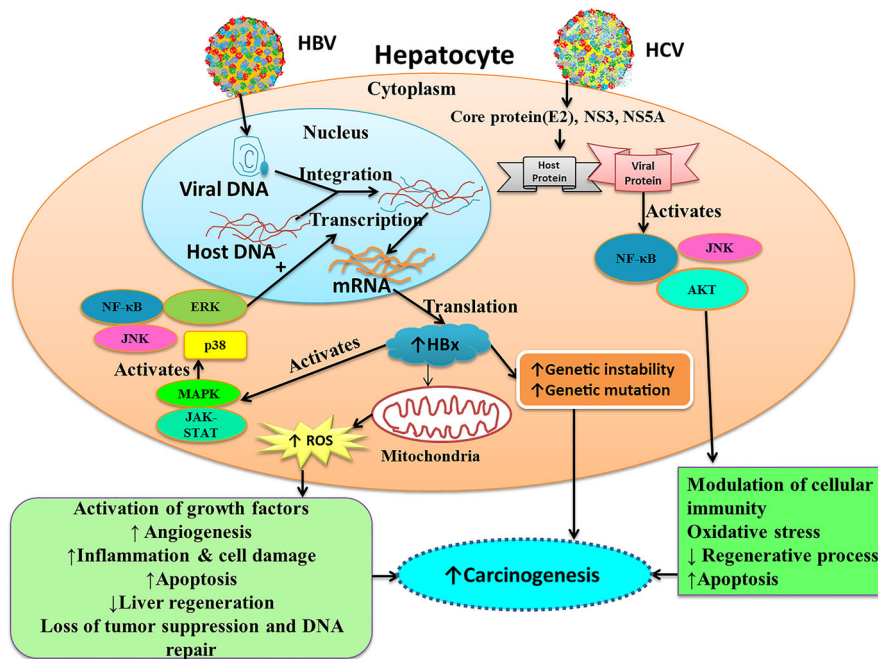


Fig. 1 Schematic representation for the pathogenesis of hepatocellular carcinoma. HBV is carcinogenic by insertional activation and genetic changes caused by increased HBx protein in cytoplasm. HBx protein also activates various transcriptional factors such as NF- κ B, JNK, and MAPK which results in enhanced viral replication and translation, resulting in more HBx formation and this cascade goes on with chronic HBV infection. HCC in case of HCV is mediated by immune suppression of the host cell by viral and host protein

interaction, activating various transcriptional pathways. DNA deoxyribonucleic acid, ERK extracellular signal-regulated kinase, HBV hepatitis B virus, HBx hepatitis B viral X protein, HCV hepatitis C virus, JNK c-Jun N-terminal kinases, JAK-STAT Janus kinase/signal transducers and activators of transcription, MAPK mitogen-activated protein kinase, NF- κ B nuclear factor-kappa beta, ROS reactive oxygen species

between regenerative and destructive processes results in chronic liver injury which progresses to fibrosis, cirrhosis, and ultimately the development of HCC (Fig. 2). The regulatory immune mechanism(s) in normal liver cells is mediated by hepatocytes and other non-parenchymal cells such as kupffer cells, dendritic cells (DCs), natural killer (NK) cells, natural killer T-cells, and liver-associated lymphocytes acting as local antigen-presenting cells (APCs). The major histocompatibility complex (MHC) class II expressing hepatocytes engage with naïve T-cells in the liver microenvironment protecting the liver from injury [7]. Spontaneous immune responses to different tumor-associated antigens are mediated by T-cells [8], humoral responses, [9] immune cell subsets, chemokines, and cytokines. Specific defects in host immune response or immune suppression contribute to the high rate of persistence of hepatitis-associated antigen(s) (hepatitis B virus) in patients [10]. The imbalance of different subsets of DCs in HBV- and HCV-positive HCC patients results in defective function of DCs and the defects in overall immune response [11, 12]. Immune suppression in the inflammatory liver cells by the neutralization of naïve lymphocytes, tolerization, inhibition of cytotoxic T-lymphocytes (CTL), T cell anergy, T-cell tolerance, and loss of

CTL function and CTL apoptosis causes unopposed development of HCC [7]. Intratumoral immunosuppression in HCC can also be mediated by CD4+ type 1 T-regulatory (Tr1) cells involving interleukin (IL)-10. Furthermore, the Tr1-dependent immunosuppression is correlated with the infiltration of plasmacytoid (p) DCs resulting in increased IL-10 production through upregulation of the inducible co-stimulatory ligand [13]. Thus, defective or suppressed immune response of the host leads to progression and development of HCC. Hence, modulating the immune response to improve it by targeting the cellular and molecular effectors involved in the pathogenesis of HCC has been used in the treatment of HCC. This systematic review discusses a comprehensive description of various molecular and cellular targets for HCC immunotherapy.

Post-treatment recurrence of HCC

Most solid tumors are managed by surgical resection, anti-cancer chemotherapy, anti-cancerous agents, and radiotherapy, but these end with a treatment failure or recurrence due to the late diagnosis of HCC. Liver transplant is another perioperative intervention for advanced cases of

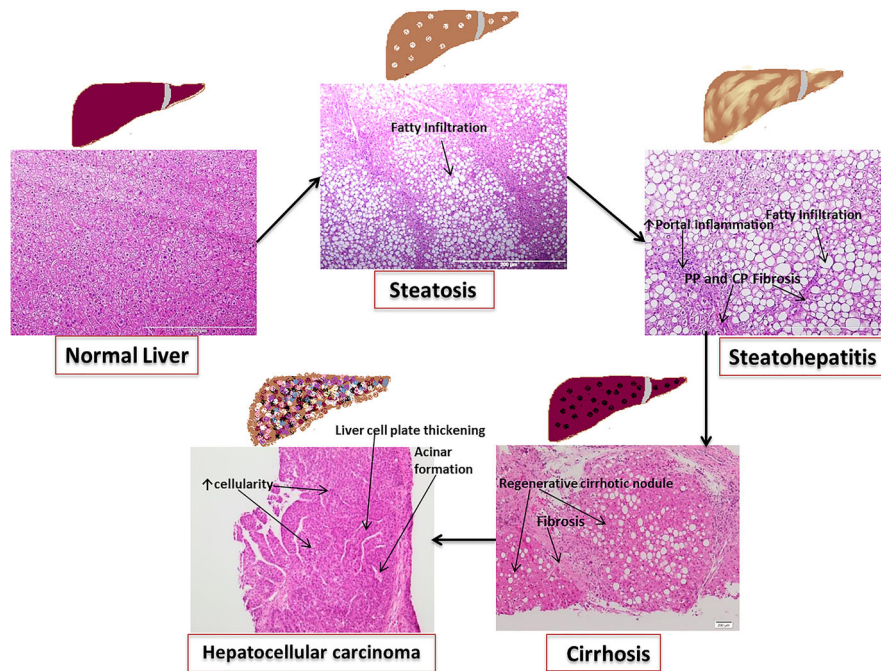


Fig. 2 Schematic and histological images showing pathological changes in various stages of liver disease. Chronic inflammation leads to oxidative stress, resistance to apoptosis, matrix deposition, disrupted architecture, parenchymal cell death, increased fibrogenesis and angiogenesis and aberrant hepatocyte regeneration (cirrhotic nodule). Steatohepatitis and fibrosis is characterized by fatty infiltration, increased portal inflammation, portal to portal (PP) and central to portal (CP) fibrosis. HCC is characterized by thickening of liver cell plates, acinar formation and increased cellularity. On higher

magnification (not shown here), hemorrhage, necrosis, higher N/C ratio, round nuclei with coarse chromatin and thickened nuclear membrane; prominent nucleoli, portal vein thrombosis, vascular invasion and mitotic figures are found in hepatocellular carcinoma (HCC). Hematoxylin and Eosin stained images of human normal liver, steatosis, fibrosis, cirrhosis, and hepatocellular carcinoma were provided by one of the co-authors, Dr. Poonam Sharma, of this article, who is a board-certified pathologist

HCC; however, there are limitations due to the insufficient number of the matched donors as well as post-transplant allograft rejection [14]. Due to the advanced stage at which most patients are diagnosed, only a small percentage is eligible for potentially curative resection, local ablation, or liver transplantation [15]. Local ablative therapies are widely used in HCC for both curative and palliative treatment to induce tumor cell death by physical destruction, radiation, or elimination of vascular supply in combination with chemotherapy. Common ablative procedures are radiofrequency ablation (RFA), laser ablation, cryoablation, photodynamic therapy, high-intensity frequency ultrasound, and percutaneous ethanol or acetic acid injection. The tumor antigens released as a result of local ablative procedures are taken up by APCs and activate a tumor-specific immune response [16], which may prevent the recurrence and can treat the metastasis.

The increased activation and cytolytic activity of tumor-specific CD8⁺ T-cell response post-RFA [17–20] and expansion of alpha-fetoprotein (AFP)-specific CD4 T-cell response post-transarterial chemoembolization (TACE) lead to amplified tumor necrosis and improved outcomes

[21]. Increased tumor-specific T-cell response has also been observed after local ablative therapy while combined with RFA and TACE treatment [22, 23], cryoablation, and high-intensity focused ultrasound (HiFU). HiFU can also cause a shift in the CD4:CD8 T-cell ratio in peripheral blood [24]. Although RFA and TACE can essentially cause a patient's tumor to act like an endogenous vaccine, there is a correlation between the expression of programmed death-ligand 1/programmed death 1 (PD-L1/PD-1), and prognosis. PD-L1/PD-1 upregulation is associated with poor prognosis post-cryoablation [25]. Thus, PD-1 blockade may enhance the immune response post-RFA [26]. In cases where the surgical resection or local ablation is not possible, TACE is used as a palliative treatment [1]. However, the impaired drug metabolism due to cirrhosis and intrinsic resistance of tumor cells to commonly used cytotoxic chemotherapy in HCC limits the use of chemotherapeutic agents [27]. Anti-viral treatment may lower the viral load but cannot decrease the risk of HCC development [28]. The drug-resistant mutation of viruses is another concern during HCC therapy [29].

Recurrence that occurs in both surgically resected and non-resected tumors indicates that dormancy of

disseminated tumor cells, dormancy of locoregional lesions, or dormancy of micrometastases may be other causes of recurrence besides blood circulation [30, 31]. The effect of surgery on mechanisms of cellular dormancy or tumor dormancy is not clear, but data suggest that partial hepatectomy can result in the growth of the remnant tumor and tumor metastasis. Epidermal growth factor (EGF), hepatocyte growth factor (HGF), vascular endothelial growth factor (VEGF), transforming growth factors (TGF)- α , TGF- β , hypoxia-inducible factor-1 (HIF-1), and matrix metalloproteinases (MMPs) are responsible for liver regeneration and mediate the modulation of tumor angiogenesis responsible for tumor growth [32, 33]. If the concept of dormant tumor cell growth is correct, then preventing progression by activating an immune response could be an adjunct therapy (immunotherapy) along with surgery for treating HCC patients with stage IV metastasis not suitable for surgical resection. Immunomodulation in such patients can induce tumor regression or delay tumor progression yielding improved therapeutic efficacy [30]. Thus, to facilitate a more efficient immune response as well as develop a potential immunotherapy, it is important to understand the molecular and cellular targets involved in HCC.

Cellular targets in hepatocellular carcinoma

Immunosuppression in the host results in progression and development of HCC and immunostimulation of various cells and molecules involved in the pathogenesis of HCC has resulted in better treatment modalities such as immunotherapy, combinational therapy, and oncolytic viral therapy. Several clinical trials of immunotherapy for HCC in humans have been conducted (Table 1) involving lymphokine-activated killer (LAK) cells, cytokine injection with interferon-gamma (IFN- γ), IL-2, IL-4, and tumor necrosis factor alpha (TNF- α). These interventions demonstrated increased tumor immunogenicity as well as non-specifically activated subsets of immune cells, infusion of APCs, infusion of activated peripheral blood lymphocytes (PBL) or tumor-infiltrating lymphocytes (TIL), and yielded an autologous tumor-based vaccine [34]. These clinical trials have shown improved outcomes, suggesting the important role of immunotherapy in treating HCC. Alteration of the immunosuppressive tumor microenvironment may convert the non-immunogenic HCC into being more immunogenic. Activating a generalized and antigen-specific immune response allows the immune system to target its attack directly on the tumor. APCs such as B-cells, macrophages, and DCs secreting MHC-I and MHC-II, can directly activate the immune response [34].

Dendritic cells

DCs are potent immune stimulatory cells specialized for initiation and shaping the immune response [45]. DCs presenting the tumor antigen to the immune system can be used in immunotherapy of HCC. Mixing the immature DCs with tumor cell lysate or purified proteins will result in processing and presenting multiple peptides in MHC class I and II. Specific gene products can be expressed by engineering plasmid DNA or viruses with DCs. Fusion of tumor cells with DCs via polyethylene glycol or electrofusion and pulsing is the strategy for using DCs in immunotherapy. This procedure utilizes large concentrations of synthetic peptides of known epitopes or mild acid-eluted mixed peptides from tumor cells where they are planted onto the DC surface. Recently via PEG or electrofusion, DCs and tumor cells have been combined to facilitate the transfer of any or all tumor proteins to a cell expressing necessary immune-activating molecules. Many studies in the murine model have supported the use of tumor lysate-loaded DCs in the treatment of tumors or improvement in anti-tumor response; however, further studies are needed [46–49].

DCs mediate immunomodulation through CTLs; however, the activity of CTLs is completely inhibited due to the downregulated expression of major human leukocyte antigen (HLA) class I molecules by HCC cells. Transfected DC-stimulated T-lymphocytes produce CTLs, which may limit the growth of HepG2 tumors. DCs transfected with HCC total RNA induce specific immune responses against HCC in vitro and in vivo [50]. Thus, DCs may be used to induce immune response via immunotherapy, but the use of DCs may be too inefficient to significantly impact the advanced stages of disease [34]. However, Zhou et al. [51] demonstrated that upregulated expression of HLA-A2 and improved sensitivity to CTL response can be achieved by transduction of Hep3B cells with adeno-associated virus (rAAV) carrying human α -fetoprotein promoter (AFPp) and the interferon- γ (IFN- γ) gene (rAAV/AFPp-IFN- γ) (Fig. 3). Superantigen-staphylococcus enterotoxin-A gene-modified tumor vaccine HepG2-SEA primed CTLs (SEA-T) has a strong stimulatory effect on the production of HepG2-specific CTL and reinforces the immune response [52]. Hyperthermia may also improve the cellular immune response to human HCC subsequent to co-culture with tumor lysate-pulsed DCs [53].

Natural Killer cells

Natural killer (NK) cells play an important role in liver immunity and account for 25–50% of the total number of liver lymphocytes. Further, the positive correlation of the number of NK cells in blood and tumor cells; the presence

Table 1 Clinical trials for immunotherapy

Clinical trial	Outcome (positive outcome/total patients)	References
Chemotherapy + IFN- γ + IL-2	Decrease in tumor size (14/20) Reduced serum AFP level	[35]
IFN- γ (sc) + GM-CSF	No clinical response	[36]
Adriamycin + LAK	Decrease rate of recurrence	[37]
Adriamycin + LAK	No benefit	[38]
Indium labeled TIL activated by IL-2 and CD3	Partial tumor response lasting for 5–10 months (2/3)	[39]
TIL activated by IL-2 and LAK cell supernatant	Improved recurrence rate	[40]
PBL activated by IL-2 and anti-CD3	Significantly improved risk of recurrence, time to recurrence, recurrence-free survival	[41]
Immature DC + GM-CSF + IL-4	Slowed tumor growth (1/2)	[42]
DC + GM-CSF + IL-4 + TNF- α + KLH	Positive DTH responses to KLH indicating successful vaccination (7/10) and mixed tumor response (1/10)	[43]
DC + TNF- α + IL-2	No tumor response	[44]

Bacille Calmette–Guérin (BCG) acts as a foreign immune stimulus; delayed-type hypersensitivity (DTH); granulocyte–macrophage colony-stimulating factor (GM-CSF) works as a growth factor for APC, primarily DCs; interferon (IFN) γ increases MHC expression; interleukin (IL)-2 activates and acts as a growth factor for T-cells; IL4 blocks macrophage development from monocytes tumor-infiltrating lymphocytes (TIL); keyhole limpet hemocyanin (KLH) activates non-antigen-specific helper T-cells; lymphokine-activated killer (LAK) cells lack tumor antigen specificity; peripheral blood lymphocytes (PBL)

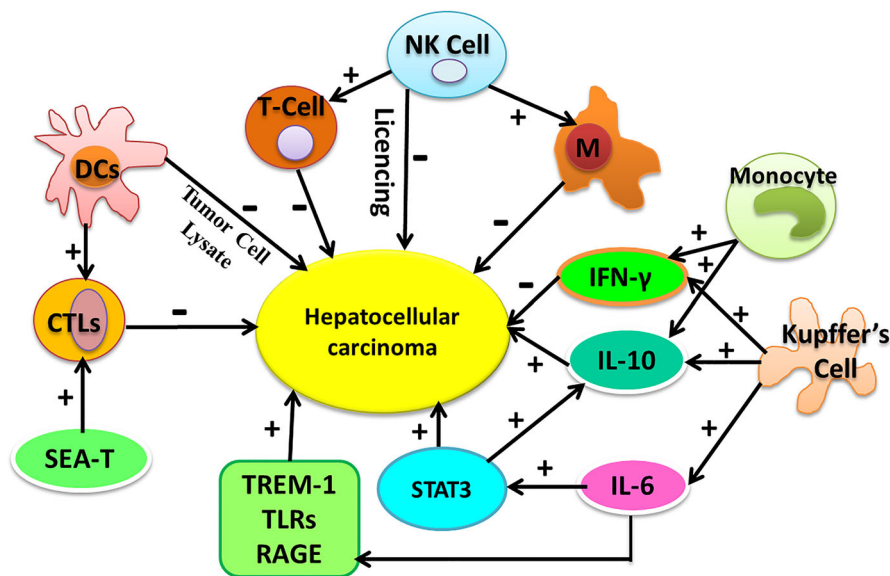


Fig. 3 Schematic representation of innate immune response of liver to viral infection and potential targets for immunotherapy in HCC (1) Natural killer (NK) cells kills virus infected cells by producing cytokines or by activating T-cells and macrophages (M). (2) Blocking signal transducer and activator of transcription (STAT) 3 with inhibitors augment NK cell function by reducing TGF- β and interleukin (IL)-10. (3) Activated NK cells through licencing enhance cytotoxic activity of NK cells. (4) Kupffer cells secrete anti-viral

cytokines (INF- γ) in response to infection. (5) Dendritic cells (DCs) combined with tumor cell lysate increase DC immune response. (6) Transfected DCs and staphylococcus enterotoxin-A (SEA-T) antigen stimulate the cytotoxic T-lymphocytes (CTLs) and increase the immune response. *RAGE* receptor for advanced glycation end products, *TREM 1* triggering receptor expressed on myeloid cell-1, *TLRs* toll-like receptors

of abnormal NK cells in blood and liver; and their functional impairment in HBV and HCV infection has been documented [54]. NK cells play an important role in

eradicating HBV from infected hepatocytes and inhibit the development of hepatitis B-related liver diseases like hepatitis, fibrosis, cirrhosis, and HCC. Disrupted cellular

cytotoxicity and cytokine production by NK cells during HBV infection indicate that restoration of NK cell function may be a potential strategy for activating the immune response. Hence, immunotherapy directed against HBV using activators of NK cells can be a potential area for further research [55]. Licensing may enhance the function of NK cells causing increased cytotoxic activity, resulting in increased overall and recurrence-free survival [56]. Similarly, blocking signal transducer and activator of transcription 3 (STAT3) in hepatocellular carcinoma cells enhances NK cell activation via reduced expression of TGF- β and IL-10 and induced expression of type I interferon (IFN), resulting in increased anti-tumor activity [57]. NK cells in human HCC *in vivo* can be augmented by hIL-15 gene-modified human NK cells, and these modified NK cells can be used for adoptive immunotherapy against HCC [58] (Fig. 3). Recently, Kamiya et al. [59] reported enhanced anti-tumor activity of expanded NK cells *in vitro* and in immunodeficient mice with a chimeric NKG2D–CD3 ζ –DAP10 receptor. Interestingly it was also reported that HCC cells treated with sorafenib remain sensitive to NK cells suggesting an additive advantage of utilizing NK cells in the presence of this multikinase inhibitor. Furthermore, IL-2-enhanced NK cells were found to decrease the pulmonary metastasis of HCC in mice [60]. Thus, improving the NK cell function via NK cell transfer or in conjugation with other therapies such as chemo-immunotherapy, gene therapy, and cytokine therapy may be effective therapeutic strategy in HCC treatment.

Lymphocytes: T-cells and T-regulatory cells

Suppression of the host immune response results in the development and progression of HCC. Decreased number and dysregulation of the T-lymphocyte has been reported in cirrhosis and HCC. Further, T-lymphocytes, both CD4+ T helper cells, and CD8+ cytotoxic T-cells are significant players in immune response and are effective in inhibiting and killing tumor cells [61]. Furthermore, T-cells are also important for B-cell maturation and their reduced number has been associated with low survival rates in HCC. Similarly, the role of Tregs, Th17 cells, and NKT cells in the pathogenesis of HCC has been discussed [62]. Thus, these cells can be targeted to enhance the immune response in HCC and may be potential targets for immunotherapy (Fig. 3).

Sorafenib, which targets multiple protein kinases, is the only effective systemic molecular-targeted treatment so far for advanced HCC, showing modest improvement in survival. However, the prolongation of survival associated with sorafenib therapy is under 3 months [63], and the median survival for patients with advanced stage, unresectable HCC is less than 1 year [15]. *In vitro*, it may

augment effector CD4+ T-cell function by Treg suppressor function elimination in peripheral blood mononuclear cells (PBMC) obtained from HCC patients in low dose [64] and decreases Th2 and regulatory T-cells in peripheral blood of HCC patients [65]. Although sorafenib has anti-proliferative and anti-angiogenic effects [66] and prolongs survival, resistance to it results in low response rates. Sorafenib resistance is possibly due to genetic heterogeneity, but the exact mechanism is not yet known. However, the roles of three major pathways (JAK/STAT, RAF/MAPK, and PI3 K/AKT) have been implicated in sorafenib resistance acquisition [67, 68]. The activation of these pathways by upregulation of epidermal growth factor receptor, platelet-derived growth factor, vascular endothelial growth factor, as well as the mutation in these pathways, contributes to sorafenib resistance [69, 70].

Increasing the number of T-cells by T-cell transfer may be another method for increasing immune response. However, this may be ineffective due to the tumor microenvironment. Chuang et al. [71] reported that sorafenib in serial low dose injections enhances the efficacy of adoptive T-cells by modulating the tumor microenvironment. Adoptive cell transfer (ACT) involves the transfer of autologous TILs and/or donor lymphocytes to modulate the immune response. Many studies have suggested that ACT can successfully treat solid tumors. ACT may be combined with chemotherapy and radiotherapy for better outcomes. Genetically engineered T-cells receptors with potent anti-tumor activity can be used in ACT. Infusion of tumor-specific T-cells has the advantage that its *ex vivo* activation avoids the immune-suppressive influence of the tumor microenvironment. The advantages of ACT along with chemotherapy and radiotherapy have been proven and may be considered as a potential strategy for HCC treatment [72–75] (Fig. 3). Another study by Hu et al. [76] reported the enhanced synergistic tumor inhibitory effect with the combination of oncolytic adenovirus expressing Hsp70 with cytokine-induced killer (CIK) cells via infiltration of CD3+ T-cells in tumor stroma. This study further supports the synergistic effect of combined anti-tumor therapy. Similarly, the case report by Li et al. [77] supports the safety and feasibility of the CIK cells in liver transplant patients to kill the residual tumor cells.

Recently, it has been suggested that glucocorticoid-induced tumor necrosis factor receptor ligation and anti-CTLA-4 mAb can improve the anti-tumor immunity by abrogating tumor-infiltrating regulatory T-cell-mediated suppression in patients with primary and secondary liver cancer [78]. Tumor-induced CD4+ CD69+ Tregs may be another potential therapeutic targets for the immunotherapy [79]. Further, T-cell-mediated immune response involves the T-cell receptor (TCR), and it has been reported that hepatitis C-associated hepatocellular carcinoma tumors can

be efficiently treated by TCR gene-modified T-cells [80]. These studies suggest that immune modulation in HCC by targeting the lymphocytes may be a potential strategy to improve the outcome of immunotherapy (Fig. 3).

Targeting the genes for the treatment of HCC

Genomic involvement in the pathogenesis of HCC has been well-documented. Gene products and an increase or decrease in expression act as diagnostic as well as prognostic factors in HCC. Gene therapy involves the induction of an immune response targeted at overexpressed protein thereby eliminating such markers. Gene therapy also aims to increase the level of proteins with reduced expression and replace non-functional or mutated genes. Several studies using gene therapy for the treatment of HCC have been conducted and have shown promising results. The injection of wild-type p53 in primary hepatocellular carcinoma showed tumor volume reduction and a significant decrease in serum alpha-fetoprotein (AFP). However, the mechanism of action of wild-type p53 needs to be investigated [81]. Similarly, treatment of HCC with allogeneic suicide gene-modified killer cells (aSGMKCs) has demonstrated a marked, rapid, and sustained regression of tumor [82, 83]. Inhibition of cellular growth and induction of apoptosis by recombinant adenovirus carrying the C-terminal fragment of human telomerase reverse transcriptase (rAdv-hTERTC27) and induction of antigen-specific T-cell proliferation along with activated cytotoxicity of T-cells by DCs transduced with rAdv-hTERTC27 in Hepa 1–6 HCC cells suggest an improved outcome of combining gene therapy with immunotherapy [84]. Cytotoxic T-cells recognize peptides with the help of TCR, leading to an immune response. But some antigen-presenting cells can also present proteins to CD8+ cells; hence, there is a need to target the immune response specifically to the tumor cells. TCR gene transfer is a promising strategy to generate stimulated AFP-specific CTLs for adoptive immunotherapy [85] and efficient treatment of HCV-associated HCC by TCR gene-modified antigen-reactive T-cells, providing another viable option of therapy [80].

When the MAGE-A family of genes is overexpressed, they become a possible target for immunotherapy. Along with MAGE-A, other genes found in HCC are members of tumor-specific “cancer-testes” gene families like NY-ESO and SSX-1. Hepatitis viral antigens and viral genes from HBV and HCV such as the HBx protein, which modulates p53 expression, can also be targeted. Non-malignant and malignant cells, as well as viral proteins can be affected by the immune system, but malignant cells should be the preferred target. Increased levels of oncogenes such as myc, fos, and ras (critical for the unregulated growth of the

tumor), and mutated and/or dysregulated tumor suppressor genes such as p53 and pRb are other potential targets for immunotherapy [34]. Antigen-specific T-cell response against HCC cells in vitro is stimulated by DCs loaded with NY-ESO-1. Hence, NY-ESO-1 may be used as a potential target for immunotherapy in advanced HCC [86]. Fujiwara et al. [87] proposed that ROBO1 is expressed at high levels in hepatocellular carcinoma and plays a role in tumor metastasis and angiogenesis. It can act as a biomarker for immunotherapy, and they found that radio-immunotherapy with the radioisotope-labeled anti-ROBO1 monoclonal antibody (^{90}Y -anti-ROBO1 MAb) is a promising treatment for ROBO1-positive hepatocellular carcinoma. Hepatocellular carcinoma-associated antigen-519/targeting protein for Xklp-2 (HCA519/TPX2) might be beneficial for T-cell-specific HCC immunotherapy as well [88].

Cellular surface receptors

Intra- and intercellular signaling is crucial for the synchronized development and vitality of multicellular organisms. This signaling cascade involves intricate signal transduction networks that run through protein receptors on the cell surface. These receptors regulate an assortment of biological functions such as cellular migration, differentiation, survival, death, or even proliferation. Impairment of the immune regulatory mechanism in HCC results in the loss of the immune system’s ability to discriminate tumor cells from unaffected hepatocytes. Studies have suggested the role of cell surface receptors in the treatment of carcinoma including HCC; however, a novel target to develop a potential drug for the treatment of HCC is yet to be determined [89, 90].

Folate receptor

Thirty percent of HCC patients are deemed ineligible to undergo harsh chemotherapies; therefore, it is imperative to cultivate unconventional therapeutic options that can target only malignant cells while avoiding toxicity in healthy organs and tissues [91]. Folate receptor (FR) is a cell surface biomarker that is overexpressed in a myriad of neoplasms, while its expression is uncommon in non-cancerous tissues [92]. Upregulation of FR has been observed in HCC; therefore, targeting this biomarker using covalent attachment to an immunotherapeutic agent could be a viable option for oncologists. This process could be utilized when the common immunotherapy target (PD-L1) is not present in the tumor. Adhesion of cancer therapy-based molecules to folic acid has demonstrated efficacy in cells that express folate receptor while at the same time avoiding uptake by healthy tissues [93]. However, the

kidneys may undergo adverse effects under these biochemical conditions. Ideally, we hope to see researchers improve the potential of using folate's capacity to transport attached molecules precisely to malignant HCC cells to transform tumors lacking immune activity into a robust immunogenic milieu. It has also been demonstrated that methods which increase IFN- α and IL-2 in the same cells in concert with immunotherapy targeting FR have a positive synergistic effect [93].

Toll-like receptors

Using toll-like receptors as an avenue to cease the development of HCC from HBV would not be a curative option for HCC but could be utilized as a preventative measure for patients infected with HBV. Heightened toll-like receptor (TLR) activity is linked to inflammatory cancers as well as viral detectors, and the increased TLR activity has a significant role in yielding a suitable immune response to antigens. The upregulation of TLR signaling has been detected in HCC suggesting that it may play a role in the inflammatory mechanisms involved with HBV progressing into HCC [94]. TLRs can sense and recognize HBV which in turn induces an immune response to combat the virus. TLR4 has been linked to the increased progression and amplified tumorigenesis of HCC; hence, exploiting TLR ligands may prove to be an effective method for treating HBV before it undergoes carcinogenesis [95]. Also, TLR2 (as a homodimer or heterodimer with TLR1/6) can detect several molecules associated with groups of pathogens (PAMPs) which successively activate NF κ B via MyD88 [95]. Consequently, fostering TLR2 expression in patients with HBV could produce an amplified immune response against HBV which would hinder the virus from ultimately developing into HCC. TLR 2 initiates the innate immune response against HBV in acute hepatitis while suppressing it in chronic hepatitis, suggesting the TLR2-dependent status of the disease (Fig. 3). Thus, targeting TLR2 may be a potential strategy for modulating the immune response and immunotherapy [96]. Phosphorothioate-modified TLR9 agonist ODN M362 has anti-proliferative and anti-tumor activity. Attenuation of proliferation and increase in apoptosis with ODN M362 in HCC makes it a potential therapeutic agent as well [97].

Triggering receptor expressed on myeloid cells-1

Triggering receptor expressed on myeloid cells-1 (TREM-1) is a biomarker that has been found to restrain inflammatory responses in several conditions and its role in many cancerous indications is currently being elucidated [98]. Because HCC is known as cancer derived from excessive inflammation, the role of TREM-1 and its explicit influence

on HCC cells have been recently investigated. In HCC cells, TREM-1 was found with statistical significance to enhance proliferation while preventing apoptosis. Pro-inflammatory cytokines IL-1 β , TNF- α , and monocyte chemoattractant protein-1 (MCP-1) have been positively correlated with TREM-1 up- or downregulation, demonstrating that this receptor acts as an inflammatory switch in the liver [98] (Fig. 3). An experiment utilizing adoptive transfer of normal Kupffer cells in mice lacking TREM-1 attenuated liver injury and even modulated malignant progression [99]. TREM-1 is an essential trigger for the activation of Kupffer cells during liver pathogenesis, bringing to light the mechanisms of how excessive inflammation reinforces the development of HCC. Taken together, TREM-1 is a viable immunotherapeutic target which could be used to attenuate Kupffer cell activation leading to a decline of multiple pro-inflammatory cytokines which would have a positive immunological effect in liver cancer cells [99].

Chemokine receptors

In addition to stellate cells and immune cells, inflamed hepatocytes, and hepatoma cells also express various chemokines attracting immune cells. CXCR4 as a receptor for the chemotactic cytokine stromal-derived factor-1-alpha (SDF-1 α /CXCL12) controls the cell migration to inflammatory foci as well as the migration of metastatic cells. CXCR4/SDF-1 α axis also plays a role in tumor initiation and progression, angiogenesis, and tumor cell survival [100, 101]. Cepeda et al. [100] described the membrane as well as subcellular presence and the intracellular trafficking pathways of CXCR4 mediating the immune response through TGF- β signaling; hence, neutralization of the CXCR4 axis may reduce the tumor progression and metastasis. Further, it has been reported that there is a correlation between higher levels of MMP-10 and enhanced expression of CXCR4 [102]. Hence, blocking MMP-10 along with CXCR4 may have a pronounced effect on the suppression of tumor progression and metastasis. Targeting the tumor-infiltrating macrophage via inhibition of CCL2/CCR2 axis in HCC resulted in immunosuppression status reversal and activation of anti-tumorous CD8+ T-cell response, suggest that blocking CCR2 may be a potential therapeutic option [103]. Similarly, the suppression of CX3CL1 by inhibitors resulting in decreased expression of immune cells such as CD56+ NK and CD8+ T-cells suggest another possible therapeutic approach for immunomodulation [104] (Fig. 4).

C-C motif chemokine ligand 2 (CCL2) is involved in recruiting CCR2+ immune cells as a mechanism to promote inflammation. This protein has been found to be prominently upregulated in HCC tumors and targeting

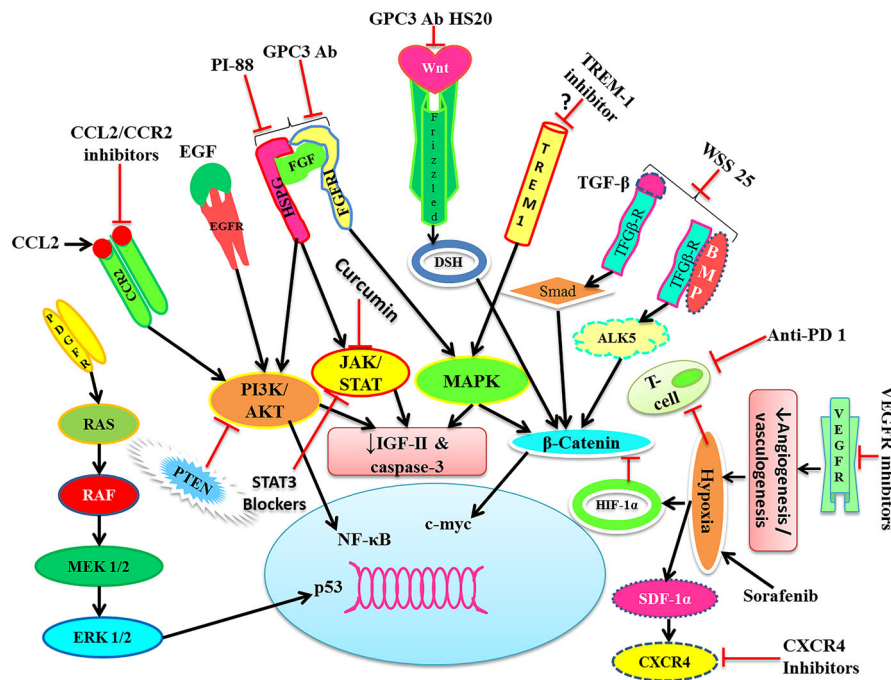


Fig. 4 Target sites for immunotherapy and mechanism of immunotherapeutic agents. *GPC-3* Glypican, *Ab* antibody, *EGF* epidermal growth factor, *HGF* hepatocyte growth factor, *VEGF* vascular endothelial growth factor, *TGF β* transforming growth factors, *HIF1 α* hypoxia-inducible factor, *JAK/STAT* Janus kinase/signal transducer and activator of transcription, *PI3K* phosphatidylinositol-3 kinase, *CCL2* chemokine, CC motif, ligand 2; *CCR2*

chemokine, CC motif, receptor 2; *caspase 3* cysteinyl, aspartyl-directed protease; *HSPGs* heparan sulfate proteoglycans, *PI-88* heparanase inhibitor phosphomannopentaose sulfate, *BMP* bone morphogenetic protein, *IGF* insulin-like growth factor, *ERK* extra-cellular signal-regulated kinase, *MEK* MAPK/ERK kinase, *PD1* programmed death 1, *SDF-1/CXCL12* stromal cell-derived factor 1, *CXCR4* chemokine (C-X-C Motif) receptor 4

CCL2-CCR2 may have a translational value in treating HCC [105]. The clinical utility of blocking *CCL2-CCR2* heterodimers was tested in knockout mice with an antibody directed at *CCL2* [106]. Inhibiting *CCL2* with anti-*CCL2* therapy triggered an immunotherapeutic-like response suppressing inflammation in the hepatocytes of these mice via the reduction of *CD11^{high}Gr1⁺* inflammatory myeloid cells, reducing the expression levels of *TNF- α* and *IL-6*, downregulation of *STAT2*, *c-MYC*, and *NF- κ B* expression, upregulation of *IFN- γ* production, and augmentation of *NK* cell infiltration in the liver. These events result in a substantial reduction in tumorigenesis, tumor burden, and overall liver damage in eight weeks of therapy suggesting it as a potential therapeutic strategy [106] (Fig. 4). *CCR5* possesses an important role in the progression of HCC, and like receptor for advanced glycation end products (*RAGE*), an antagonist monoclonal antibody-based therapy directed at *CCR5* has the potential in the prevention and treatment of HCC [107]. The recruitment of macrophages to hepatocytes was significantly reduced in mice lacking *CCR5*, which in turn reduced inflammation in the liver. Knocking out *CCR5* in murine models yielded a noteworthy reduction in tumor size and rate of incidence [107]. The roles of *CCL15/CCR1*, *CCL17*, and *CCL20/CCR6* in the migration, invasion, progression, and prognosis of HCC suggest

that these chemokines may be useful in therapeutic targets [108–112].

Receptor for advanced glycation end product

RAGE is primarily involved in the tissue where inflammatory destruction has taken place, acting as an auxiliary to inflammatory responses when dispatched by the immune system [113]. This mechanism is typically observed when *RAGE* proteins encounter damage-associated molecular pattern proteins (*DAMPs*). Augmented *RAGE* expression has been detected in many solid tumor indications and recent investigations have shed light on its critical role in carcinogenesis and metastasis; however, the level of significance remains undetermined. *RAGE* has been shown to be involved in regulating oval cell activation and tumor progression in liver cancer caused by inflammation which would make blocking the *RAGE* receptors a potential immunotherapeutic avenue for HCC patients [113, 114] (Fig. 3).

In sum, there are several feasible immunotherapeutic targets on the cell surface of HCC tumors; however, it should be noted that tumors have evolved multiple immune-based escape mechanisms. These pathways involve cells like *Tregs* and myeloid-derived cells which

possess the capacity to suppress immune responses [114]. It has been shown that these suppressor molecules mask tumor-specific immune responses in patients with HCC. Due to these escape mechanisms built into the tumors, novel immune-based therapeutic avenues including dendritic cell-based therapies have been investigated and yielded encouraging data in HCC patients [114]. Presumably, these methods should be delivered in concert with a drug directly targeting suppressor cells in HCC to potentially improve treatment efficacy.

Molecular Targets for treatment of HCC

Proteins and proteoglycans

Alpha-fetoproteins

Most of the studies on immunotherapy have used either cytokine-based or antigen-based approach [115, 116] and have proposed that immunotherapy can induce a tumor-specific immune response but lacks the clinical efficacy. Sensitivity, specificity, and self-regulation of the immune system to find and eradicate tumor cells from the body can be achieved by immune-based therapy. The activation of HCC-specific immune response can be achieved by targeting the AFP, a tumor-associated self-antigen and other such antigens and the HCC-specific genes [34]. Alpha-fetoprotein, a serum marker for HCC diagnosis, is found in high levels in serum, which is highly indicative of the presence of HCC [117] with a sensitivity of 25–65% and specificity of 79–95% [118] and AFP can be targeted to generate tumor antigen-specific immune responses to HCC. Strategies used to induce an immune response to this self-antigen include AFP vaccination, peptide-pulsed DC, plasmid DNA, AFP-derived peptides, antigen-engineered DC, and AFP-based prevention strategies in animal models, *in vitro* T-cell cultures, and clinical trials [34, 119–121]. Alpha-fetoprotein is a tumor marker for HCC, and it makes it a choice for HCC tumor cell target. AFP has been suggested as a target for antibody-based therapy, but as the AFP peptides are processed by the cell and can be presented to both CD8+ and CD4+ cells, the results of such studies are inconclusive [34].

Glypican 3

Glypican 3 (GPC3), a member of the glypican family of heparan sulfate (HS) proteoglycans, attached to the cell membrane is tumor specific and important for cell proliferation [122]. GPC3 is a carcinoembryonic antigen, and patients with GPC3-positive HCC have poor 5-year survival rates, linking it to poor prognosis [123]. GPC3 is

found overexpressed in nearly 80% of HCC [124, 125], can be used as a serum marker, and also differentiate between high-grade dysplastic nodules from an early HCC by immunostaining [126]. Hence, it is a potential new target antigen for the immunotherapy. Anti-GPC3 antibody and peptide-based vaccines for the treatment of HCC are in the pre-clinical and clinical phase of development [127, 128]. The potential anti-tumor activity of recombinant fully humanized monoclonal antibody GC33 that binds to human GPC3 through antibody-dependent cellular toxicity has been reported. Treatment with humanized GPC3 antibody enhances the susceptibility of HCC to chemotherapy [129]. Pre-clinical studies have suggested the combination of anti-GPC3 antibody and sorafenib, to be more effective in inhibiting tumor progression than sorafenib alone [7].

Human heavy-chain variable domain antibody HN3 inhibits the proliferation of GPC3-positive cells and HCC xenograft tumor growth via cell-cycle arrest at G1 phase through Yes-associated protein signaling [128]. Combining NIR-PIT (near infrared photoimmunotherapy) using IR700-HN3 with fluorescence-guided surgery may result in a superior therapeutic outcome [130]. Gao et al. [131, 132] reported monoclonal antibody HS20 that inhibits the cell proliferation through Wnt/ β -catenin signaling and HGF-mediated migration and motility by targeting the heparin sulfate chains of GPC3. Sun et al. [133] reported the role of GPC3 suppression in inhibiting the cell proliferation by arresting cell-cycle progression at G1 phase through TGF- β signaling pathway activation suggesting GPC3 as a target for HCC immunotherapy. Recently, Zaghoul et al. [134] suggested blocking of HSPG/SULF-2/IGF-II axis as a mechanism of action of anti-GPC3 antibody, leading to decreased gene expression of IGF-II and caspase-3 and the activity of sulfatase-2. The potential role of T-cells expressing GPC3-targeted chimeric antigen receptor in eliminating GPC3-positive cells in HCC has been suggested by Gao et al. [135]. Anti-GPC3 antibody such as YP7 and MDX-1414 has been reviewed in the literature [136]. Li et al. [137], using mouse model, reported that specific and effective cellular anti-tumor immunity against GPC3 can be elicited by GPC3 DNA vaccine, and Luo et al. [138] proposed that GPC3 expression in the mouse ovarian cancer increases F4/80+ CD86+ macrophage (M1) proportion and induces GPC3-specific CD8+ T-cell immune response through these macrophages, prolonging survival. These studies have demonstrated the mechanism of action and the chemopreventive effect of anti-GPC3 therapy by targeting the GPC3 for HCC therapy (Fig. 4). However, the therapeutic potency and hepatoprotective effect of anti-GPC3 antibody, its stability, its role in the modulation of GPC3 functions, and the specificity for the cancer-associated antigen involving signaling pathways remain to be elucidated.

Heparan sulfate

The basis of heparan sulfate (HS)-based immunotherapy is the emerging important role of heparan sulfate proteoglycans (HSPGs) in tumorigenesis, progression, and metastasis of the tumor through HS chain of HSPGs [139]. Heparanase inhibitor phosphormannopentaose sulfate (PI-88) exhibited anti-tumor, anti-metastasis, and anti-recurrence activity in HCC in pre-clinical trials [140], and it has been reported that upregulation of heparanase by overexpression of early growth response 1 enhances the sensitivity of HCC cells to PI-88 resulting in inhibitory cell proliferation and migration effect after hepatectomy [141]. Liu et al. [142] suggested that PI-88 is safe and well tolerated at 160 mg/d, and confer the most significant survival advantage. WSS25 is another HS-mimetic which inhibits the growth of xenograft HCC cells by decreasing the angiogenesis via bone morphogenetic protein (BMP)/Smad/Id1 signaling blockade [143] (Fig. 4).

Cytokines

The development of hepatocellular carcinoma is a highly complex multi-step pathologic process involving numerous cell, chemokine, and cytokine signaling. The role of Th1 cytokines including IL-1 α , IL-1 β , IL-2, IL-12p35, IL-12p40, IL-15, Th2 cytokines such as IL-4, IL-8, IL-10, and IL-5, and non-ILs including TNF- α and IFN- γ in the pathogenesis of HCC has been discussed [144]. Further, the pathogenic role of imbalance between pro-inflammatory and anti-inflammatory cytokines in the development of HCC suggests that targeting the cytokines may modulate the course of disease [144]. The role of cytokines in modulating the signaling pathways involved in the pathogenesis of HCC and the use of cytokine levels in the assessment of recurrence, prognosis, and post-treatment outcome suggest cytokines as potential targets in the immunotherapy of HCC [145, 146]. The immunomodulatory and anti-tumor activity of TNF α , type I or type II interferons, IL-2, IL-12, IL-15, IL-18, IL-21, IL-23, IL-27 has been discussed in literature [147]. The role of activated cytokine-induced killer (CIK) cells (CD3⁺/CD56⁺ and CD3⁺/CD56⁻ T-cells and CD3⁻/CD56⁺ natural killer cells) as adjunct immunotherapy with curative treatment in prolonging the recurrence-free survival of patients after curative therapy for HCC suggests the role of cytokines in the treatment of HCC [77, 148–150]. Improved 1- and 2-year overall survival, overall response rate, disease control rate, and a better quality of life with the combination of DC-CIK immunotherapy and TACE or TACE plus local ablation therapy for patients with HCC indicate the role of cytokines in HCC immunotherapy [151]. The use of recombinant IL-10 therapy in the long term

decreases the disease activity, but was associated with increased HCV viral burden via alterations in immunologic viral surveillance [152].

MicroRNA

MicroRNAs (miRNAs) are conserved endogenous small non-coding RNAs that regulate gene expression by interacting with the 3'-untranslated region (3'-UTR) of protein-coding mRNA. By recruiting the RNA-induced silencing factor complex (RISC), miRNAs binding generally leads to translational suppression and/or degradation of the target transcript [153–155]. Recently miRNAs have emerged as key factors involved in several biological processes, including differentiation, cell proliferation, metastasis, and tumorigenesis [156]. MiRNA biogenesis itself involves multiple steps, and each of these steps can be affected or altered to impact the amount of produced mature miRNA. Epigenetic mechanisms (e.g., histone deacetylation and DNA methylation) can result in miRNA silencing [157]. For instance, Furuta et al. [158] showed that methylation of miR-124 and miR-203 genes in HCC cell lines silenced their expression. Besides the possible alterations in the miRNA processing, miRNA polymorphism in the form of single nucleotide polymorphism (SNP) can also be associated with an increased risk of HCC [157]. Several evidences have shown that miRNAs may act as oncogenes or tumor suppressors by directly or indirectly controlling the expression of key genes involved in cancer-associated pathways [159]. One gene can be targeted by several miRNAs, and one miRNA may regulate the expression of many genes. Aberrant expressions of miRNAs have been widely reported in human cancers with both upregulation and downregulation detected in neoplastic cells compared with their normal counterparts [160–162].

Several miRNAs are dysregulated in HCC and this results in changes in the expression profile of target genes involved in HCC onset and progression (Table 2). These specific miRNAs may serve as potential biomarkers for diagnosis, monitoring, prognosis, and therapeutic targets in patients with HCC. Cellular miRNAs can be released into the circulation and can be detected in most body fluids. Circulating miRNAs can, therefore, correlate with disease activity and progression. Several recent studies reported that miRNAs are stably detectable in plasma and serum [162, 163]. The most commonly used methods to detect miRNA are RT-qPCR, microarray, and next-generation sequencing (NGS). The high stability of miRNAs in circulation makes them very useful especially for early detection of HCC, even in pre-symptomatic diseases [164]. For instance, the circulating miR-21 [165, 166], miR-221 [166], and miR-223 [160] were found to be upregulated in the serum of HCC patients associated with HBV or HCV.

Table 2 Circulating miRNAs with potential biomarkers in HCC patients

miRNA	Dysregulation/expression in HCC	Target gene/s	Mechanism	References
miR-18	Up	c-Myc, E2F	Angiogenesis	[153, 184]
miR-21	Up	PTEN, RHOB, PDCD4	Proliferation Metastasis	[153, 185] [177, 186]
miR-92-1	Up	c-Myc, E2F	Angiogenesis	[153]
miR-101	Down	SOX9, Mcl-1, EZH2, EED	Clonal formation DNA methylation, apoptosis	[177, 185] [184]
miR-122	Down	Cyclin G1, Bcl-w, TACE, Wnt1	Apoptosis, angiogenesis, metastasis	[153, 177]
miR-139	Down	ROCK2, c-Fos	Metastasis	[184, 185]
miR-181b	Up	TIMP3	Cell growth, metastasis	[177, 185]
miR-195	Down	CDK6, Cyclin D1, LATS2	Apoptosis, cell cycle	[153, 157, 185]
miR-199a	Down	mTOR, c-Met, PAK4	Proliferation, metastasis, cell growth	[177, 186]
miR-221	Up	p27, p57, Arnt, CDKN1B	Apoptosis, proliferation, angiogenesis	[153, 177]
miR-222	Up	PTEN, AKT	Metastasis, angiogenesis	[153, 157]
miR-223	Down	STMN1	Proliferation	[177, 185]
miR-224	Up	RKIP, CDC42, CDH1, BCL-2, API-5	Apoptosis, metastasis, proliferation	[153, 185]
miR-338-3p	Up	β -catenin, VEGF, MACC1	Angiogenesis	[187]
miR-1299	Down	CDK6	Proliferation	[188]

At the same time, serum levels of miR-1, miR-25, miR-92a, miR-206, miR-375, and let-7f were also significantly elevated in HCC patients [167]. Serum miR-15b and miR-130b levels were also found to be upregulated in HCC with high sensitivity of 98.3 and 87.7%, respectively [153, 168]. Therefore, circulating miR-15b and miR-130b are potential biomarkers for HCC in early stage where patients may have low AFP levels despite the presence of the tumor [153]. In one study, the combination of miR-16 with chemical biomarkers (AFP), Lens culinaris agglutinin-reactive AFP (AFP-L3), and descarboxyprothrombin (DCP) allowed the detection of HCC cases with a high specificity even when the tumor is <3 cm in size [169].

The testing and interpretation of qualitative miRNA in a clinical setting is complex, firstly because of the variability in the technical procedure, from the method of sampling (open biopsy, cytology), time to freeze the tissue and procedure of freezing, RNA isolation, and method of detection. Secondly, HCC patients with a single underlying etiology (alcohol or viral) may yield different results than multiple etiologies (alcohol and viral). Thirdly, the stage of the disease should also be considered, although miRNA dysregulations occur from an early stage [157, 170]; it is not clear how miRNA expression changes during disease progression. Finally, in a clinical setting, the use of appropriate controls for miRNA is critical as HCC is often accompanied by viral hepatitis, cirrhosis, or other underlying liver conditions [171]. It is important to ensure that

patients and control tissue used for normalization are matched not only by age and sex, but also by the etiology and severity of the underlying liver disease [153].

The use of miRNA-targeted treatment is in early stage but holds promises for HCC therapy by potentially affecting several target genes with only one miRNA. In HCC, miRNA associated with tumor suppressors are downregulated and the one associated with oncogenes are upregulated during tumor development and metastasis. Targeted approaches for miRNA replacement therapy have been developed using miR-26a [172], miR-122 [173], and miR-124 [174] in a HCC mouse model. For instance, restoration of tumor-suppressive miR-122 makes HCC cells more sensitive to Sorafenib treatment by downregulating drug resistance genes [153, 175]. In contrast, suppression of oncogenic miR-221 in HCC resulted in prolonged survival with reduction in the number and size of tumors in animals [176]. Furthermore, no toxicity was observed when miRNA-targeted therapy was used to treat HCC in a mice model [177]. MiRNA has been also shown to affect the sensitivity of tumors to anti-cancer drugs. Overexpression of miR-21 [178] and miR-181b [177, 179] induced resistance to interferon- α /5-fluorouracil (5FU) combination therapy and doxorubicin treatment in HCC. Interestingly, HCC cells transfected with anti-miR-21 were significantly sensitive to chemotherapy with combined IFN- α and 5-FU [153, 178].

The recent advances in gene therapy create more opportunities for miRNA-based gene therapy applications.

Such therapy could even be used in conjunction with chemotherapy regimen. The main challenge to these treatments is the delivery of the synthetic effector molecule to their target genes in a specific tissue [157]. Gene therapy using viral-delivered miRNAs provides new therapeutic approach to deliver specific miRNAs via viral vector to tumor cells. However, this method raises safety concerns in clinical use as these viruses integrate their genetic material into host genome. No virus-delivered miRNA-based gene therapy has been tested in clinical trials yet [157]. MiRNA antagonists are a class of chemically engineered oligonucleotides that cause transient suppression of target gene expression. To prevent rapid degradation, miRNAs are conjugated to improve stability of effector molecules to the target cells. Stable nucleic acid lipid particle (SNALP) formulations composed of a lipid bilayer and a PEG-lipid derivative have been used as a delivery system for miRNA. When miRNAs incorporated SNALPs, they protect them from degradation, prevent immunostimulation, and facilitate their uptake in endosomes [157, 180]. Miravirsen (SPC3649) is a miR-122 antagonist drug that shows extensive anti-viral effects among all HCV genotypes and a high barrier to drug resistance. This treatment was well tolerated among patients with chronic hepatitis C that led to the suppression of HCV viremia [181, 182]. However, resistance has been reported due to mutations in miRNA binding site in HCV 5'-UTR [183]. The field of miRNA-targeted therapy is quite recent and more research still needs to be done to explore the potential benefits in clinical practice.

Multiple target approach

Combinations of the therapeutic agents for HCC are more effective than the therapy with a single therapeutic agent due to the synergistic effect (Table 3). Combination therapies may consist of gene therapy with immunotherapy, gene therapy with oncolytic virotherapy, oncolytic virotherapy with gene and chemotherapy, and gene therapy with oncolytic virochemoradiotherapy.

Oncolytic virus therapy

The selective infection, within tumor replication, and the destruction/eradication of tumor cells via oncolysis make oncolytic viruses (OV) an important strategy in HCC therapy. Oncolytic vesicular stomatitis virus (VSV) and Newcastle disease virus (NDV) prolonged the survival in orthotopic HCC by tumor-specific cell lysis in the rat model [195–197], but due to the spontaneous malignant transformation in the immune-competent setting in patients, it is difficult to transfer the efficacy of the

treatment by OVs to clinics. The microenvironment of the liver contributes to the inhibitory effect on OV therapy and limits the successful delivery and propagation of the virus to tumor tissue. The innate immune defense by NK cells against the virus, the production of anti-viral cytokines (IFN- γ and TNF- α) from non-parenchymal cells (kupffer cells, stellate cells), and the sinusoidal endothelial cells that provide a platform for non-specific uptake and a barrier for viral spread combine to limit the success of OV therapy. Additionally, the presence of chronic inflammation, altered hepatic blood flow, constitutive cytokine expression, and extracellular matrix deposition pose the further limitation to OV therapy [198].

The development of novel strategies with enhanced viral replication, propagation, and virus-mediated anti-tumor response in the complex setting of hepatocellular carcinomas is currently needed for more effective and efficient OV therapy. To be an efficient OV, the virus must reach and infect the intended tissue, should replicate and spread efficiently in the tissue, and the inserted transgene must be chosen rationally to provide maximum benefit for the treatment of the cancer [199]. Arming the therapeutic gene, antibody, and chemotherapeutic agent with OVs to enhance the anti-tumor activity synergistically is an emerging prevalent strategy to improve the oncovirotherapy of cancer. Armed OVs combine antibody therapy, gene therapy, immunotherapy, and oncovirotherapy, making OVs a potential and promising agent for HCC treatment [66, 200, 201] (Table 4).

Despite the improved outcome, low intra-tumor titers of OVs may affect the treatment and hamper the tumor lysis due to insufficient tumor targets and strong anti-viral immune response of the host. Hence, there is a need to improve the intra-tumor viral titers. Almstätter et al. [220] proposed the concept of combining OVs with magnetic nanoparticles for effective delivery, leading to higher titers of OVs in tumor tissue. Enhanced tumor necrosis and synergistically prolonged survival have been reported by combined transarterial viroembolization of VSV and degradable starch microspheres to produce the same effect of apoptosis, anti-angiogenesis, and induction of anti-tumor immunity in HCC [221]. Similarly, folate-conjugated chitosan nanoparticles (FA-CS-NPs) with mouse interferon- γ -inducible protein-10 (IP-10) plasmid showed enhanced anti-tumor activity with inhibition of tumor growth and prolonged the survival time, suggesting the potential role of nanoparticles in effective delivery [222].

Another strategy for effective virotherapy may be the suppression of the host immune response to facilitate the replication and propagation of OVs. Suppression of immune response limited to the area of viral replication is the best strategy, alleviating the concern of general immune response suppression, and can be achieved by

Table 3 Combination therapies for hepatocellular carcinoma and their outcome

Combination therapy	Study outcome	References
Secondary lymphoid tissue chemokines (SLC) and anti-CD25 mAbs	Altered tumor microenvironment, systemically optimized percentages of Tregs, CD8+ T-cells and CD4+ T-cells in peripheral immune organs	[189]
Dendritic cell–cytokine-induced killer cell (DC-CIK) immunotherapy with chemotherapy	More effective than alone TACE, improve patients' progression-free survival time, quality of life but not the overall survival time	[190]
Endothelial progenitor cells (EPCs) loaded with cytosine deaminase (CD) plus 5-fluorocytosine (5-Fc)	Stronger carcinoma growth suppression, enhanced inhibition of growth of endothelial cells, induction of apoptosis in carcinoma cell	[191]
GM-CSF with bifunctional small hairpin RNA interference (bi-shRNAi)	Increased survival with autologous whole-cell tumor cell immunotherapy (FANG therapy, phase I trial)	[192]
Fluvoxamine, glycyrrhizinic acid, and dehydroepiandrosterone	A psycho-neuro-immunological treatment, improvement in liver function, and reduction in AFP levels	[193]
Dendritic cell vaccine plus-activated T-cell transfer (ATVAC)	Prevent the post-operative recurrence, improves long-term survival in DTH positive patients	[194]
Interleukin-2, Bacillus Calmette–Guerin, and melatonin	Effective for the treatment of recurrence in HCC	[30]

incorporating anti-inflammatory protein-encoding genes directly into the OVVs [223]. Production of chemokine-binding proteins (CKBPs) by viruses to counteract antiviral immune response to promote their own growth and to enhance the potency may be another field of study in using OVVs. High affinity and broad range vCKBPs, M3 gene from murine gammaherpesvirus-68, and recombinant VSV vectors encoding for the equine herpes virus-1 glycoprotein G are examples. Stimulation of the host's immune response against tumor cells using OVVs is another strategy to treat HCC, mainly in tumors where viral replication is not possible due to unfavorable conditions. OVVs can break the tolerance of liver cells and enhance the immunogenicity of the tumorous microenvironment of the liver [198]. Interleukin (IL)-12 has potent immunostimulatory activity and anti-angiogenic properties. He et al. [224] reported the enhancement of tumor growth suppression, apoptosis induction, massive accumulation of immune cells (CD8+ T leukocytes, macrophages, and DCs), and reduction in angiogenesis in the tumor tissue with IL-12 combined with recombinant adenovirus expressing HBx (Ad-HBx-mIL-12), suggesting the potential benefit of combining virotherapy with immunotherapy therapy.

Other strategies for virotherapy with immunotherapy of HCC that require further research work include adoptive transferring of immune cells together with OVVs, incorporation of "T-cell engagers" into OVVs, and systemic use of oncolytic NDV followed by DCs pulsed with viral encolyte intradermal vaccination. Subsequent administration of two different recombinant OVVs for priming the immune response through tumor-associated antigen expression by the first and boosting secondary response by the second leads to robust tumor-specific immunity. OVVs cannot be administered systemically due to virus inactivation by

blood components; hence, there is a need for a carrier that can save the virus from inactivation. PEGylation may serve this purpose [198].

Targeting the immune checkpoints

Effective and successful host immune response via effective CTL activity is needed to protect the liver from inflammatory injury, along with a balance between the positive and negative signals from the T-cell receptors and co-regulatory ligands. Any defect in the host immune response will cause liver cell injury. Evasion of the host immune response by cancer cells may be due to the protective microenvironment of the tumorous liver [225] or by the complex interaction between the tumor cells and the liver cells such as fibroblasts, regulatory immune cells, endothelial cells, and pericytes, IL-10, TGF- β , Fas, and other membrane-bound molecules [226]. Immune checkpoint inhibitor therapy can be a potential target for further research work. PD-L1, a T-cell co-stimulatory molecule of B7 family, is found overexpressed in HCC [227] and is related to tumor aggressiveness and poor prognosis [228, 229]. Stimulated B-cells, T-cells, and myeloid cells express PD-1 a receptor for PD-L1. PD-L1 ligation of PD-1 is immunosuppressive; hence, inhibition of this interaction may be a strategy for immunotherapy and may enhance HCV and HBV clearance along with preventing HCC recurrence [230, 231]. Blocking the ligands CD80 and CD86 and CTLA4 by anti-CTLA-4 antibody results in the promotion of T-cell activation and may be used to enhance the immune response [232, 233]. Suppression of both T and NK cells [234], along with induction of CD4+ regulatory T-cells in HCC patients, is done by a subset of immune suppressor cell called myeloid-derived suppressor cells

Table 4 Oncolytic virus therapy for HCC

Virus Species/ references	Therapeutic oncovirus	Modification	Type of combination therapy	Advantageous effect
New Castle Disease virus (NDV) [200]	rNDV-18HL	Recombinant NDV carrying intact cHAb18 gene, cHAb18-mouse-human chimeric HAb18 antibody against tumor-associated antigen CD147	OV-antibody therapy	Inhibition of invasion and migration of HCC cells Increased anti-tumor efficacy
New Castle Disease virus (NDV) [202]	rNDV-IL-2- TRAIL	Recombinant NDV with IL-2 and TNF-related apoptosis inducing ligand, Integration of tumor specificity of NDV, immune regulation of IL-2 and inducing apoptosis of TRAIL	OV-immunotherapy	Significant enhancement of anti-neoplastic activity Better therapeutic efficacy than rNDV-IL-2 or rNDV-TRAIL alone
New Castle Disease virus [203]	rLaSota/IL2	Insertion of gene coding for IL-2 in rNDV (LaSota strain), uniquely remember a pathogen through the generation of memory T cells, enhances the immune stimulatory properties of the virus	OV-gene therapy	Effective regression of tumors, protect the host in the event of reinfection and form adaptive immune system
Adenovirus [201]	ZD55-XAF1 with cisplatin	Oncolytic adenovirus vector ZD55 carrying XAF1 cDNA	OV-gene-chemo therapy	Decreased proliferation and tumor growth inhibition, enhancement of tumor cell apoptosis, decreased toxicity of cisplatin by decreased dose
Adenovirus [204]	QG511-HA-Melittin	Triple-controlled cancer-selective oncolytic adenovirus with melittin gene, hypoxia-response element (HRE)-AFP promoter controlling viral E1a expression targeting AFP-positive cancer cells, E1b-55 kDa gene deletion to target p53-deficiency cancer cells	OV-gene therapy	Tumor-selective replication capability in AFP-positive tumor cell lines Strong inhibitory effect of AFP-positive HCC cell proliferation
Adenovirus [205]	GOLPH2-regulated GD55	Replacement of endogenous E1A promoter by GOLPH2 promoter to regulate E1B-55kD- deleted ZD55	OV-gene therapy	Higher adenovirus replication and infectivity for liver cancer cells, Significant growth-suppressing effect, More sensitive to HCC cells and more efficient anti-tumor effect
Adenovirus [206]	AD55-Mn-SOD	Dual-regulated oncolytic adenovirus Ad-AFP-E1A-E1B (Δ 55 kDa)-Mn-SOD, activation of caspase apoptotic pathway	OV-gene therapy	Tumor-specific and more cytotoxic activity
Adenovirus [207]	Ad5-HC, Ad5-AFP (IRES)	Combined with 5-FU, doxorubicin, and paclitaxel	OV-chemotherapy	Enhanced sensitivity of the tumors to chemotherapy and enhanced anti-tumor effect
Adenovirus [208]	hTERT-Ad	Strong downregulation of Mcl-1 expression, Increased sensitivity of HCC for TRAIL and chemotherapy-mediated apoptosis	OV-chemotherapy	Overcame the resistance of HCC against TRAIL and chemotherapy
Adenovirus [209]	hTert-Ad	Combined with bortezomib	OV-immunotherapy	Disruption of UPR via proteasome inhibition, enhanced ER stress-induced apoptosis, improved local oncolysis and anti-tumoral immunity
Adenovirus [210]	Ad-199T	Replication competent oncolytic adenovirus, by introducing copies of miR-199 target sites within the 3'-UTR of E1A gene	OV-gene therapy	Tumor-specific viral expression and replication, anti-tumor activity with reduced hepatotoxicity
Adenovirus [211]	Ad Δ E1b Δ VVA+ 2'AP	Adenovirus with deletion of E1b and the VA-RNA genes combined with 2-aminopurine	OV-gene therapy	Enhanced oncolytic activity without reducing selectivity

Table 4 continued

Virus Species/ references	Therapeutic oncovirus	Modification	Type of combination therapy	Advantageous effect
Adenovirus [212]	Ad. SP-E1A-E1B (D55)-TSLC1 (SD55-TSLC1)	Insertion of tumor suppressor TSLC1 gene into the dual-regulated oncolytic adenovirus vector Ad. SP-E1AE1B (D55)	OV-gene therapy	Increased selective viral replication and enhanced anti-tumor activity
Adenovirus [213]	Ad. eSurp-hSulf1	Survivin promoter-regulated oncolytic adenovirus with human sulfatase-1 (hSulf-1), radiation-inducible enhanced promoter (eSurp) and combined with I^{131} -metuximab monoclonal antibody	OV-gene-chemo-radio therapy	Potent tumor inhibition and excellent anti-cancer outcome
Vaccinia Virus (VACV) [66]	GLV-1h68 Phase I clinical trial	GL-ONC1 vaccinia virus, recombinant vaccinia virus derived from the vaccine strain used to eradicate smallpox	OV-gene therapy	Effective for sorafenib resistant/treatment failure HCC Cytoplasmic replication of VACV prevent genomic recombination with host
Vaccinia Virus [199]	GLV-2b372 Phase I/II clinical trial	Derivative of the less attenuated L1VP 1.1.1 isolate	OV-gene therapy	Efficient anti-tumor activity Reduces tumor growth as well as tumor size
Vaccinia Virus [214]	JX594 Phase II clinical trial	Vaccinia GM-CSF/TK-deactivated Virus, oncolytic poxvirus derived from Wyeth strain vaccinia virus	OV-gene therapy	Enhanced tumor-specific immunity by JX594-derived granulocyte-macrophage colony-stimulating factor (GM-CSF)
Vaccinia virus [215]	Pexa-Vec (JX-594), Phase I/II clinical trial	Induction of GM-CSF-driven tumor-specific immunity	OV-gene-immunotherapy	Safe in pediatric population
Recombinant vesicular stomatitis virus (VSV) [216]	recombinant VSV	Activated HSCs infected with rVSV-LacZ mediates cytotoxicity	OV therapy	VSV cause cell death specifically in activated hepatic stellate cells, safe and effective for treating HCC with fibrosis
VSV [217]	VSV with NSC74859	Inhibition of STAT3 by NSC74859	OV therapy	Enhanced reduction in cell proliferation
Parvovirus (PV) [218]	Recombinant H-1 PV	Induced expression of PML	OV-gene/chemo therapy	Suitable for p53-impaired/negative tumors
Measles vaccine viruses (MeV) [219]	MeV-SCD	MeV armed with Super-cytosine deaminase (SCD), Induction of apoptosis-like cell death not dependent on intact apoptosis pathways	OV-gene therapy	Significant enhancement of the cytotoxic effect, novel therapy for resistant tumors

GOLPH2 a Golgi membrane glycoprotein GP73, *hTert-Ad* human telomerase reverse transcriptase promoter-regulated adenovirus, *hSulf-1* human sulfatase-1 gene suppresses the growth factor signaling pathways and inhibits the proliferation of cancer cells and enhance cellular sensitivity to radiotherapy and chemotherapy; *IL-2* interleukin-2 stimulates T-cell propagation; *I¹³¹-mab* I^{131} -metuximab is a monoclonal anti-HCC antibody, *Mn-SOD* manganese superoxide dismutase, *PML* promyelocytic leukemia protein, *STAT3* signal transducer and activator of transcription 3, *TRAIL* tumor necrosis factor related apoptosis inducing ligand, *UPR* unfolded protein response

(MDSC), which are generally higher in frequency in HCC; and it has been demonstrated that there is a correlation between outcome and MDSC frequencies. Poor prognosis in patients undergoing surgical resection has also been correlated with tumor-infiltrating CD4+ regulatory T-cells [235, 236].

VEGF, a promoter of vasculogenesis and angiogenesis, can act as an immunosuppressor by promoting

immunosuppressive cell infiltration and enhances the immune checkpoint molecule expression. VEGF inhibition results in increased tumor hypoxia by decreased angiogenesis, hepatic vascular regression, and HIF-2 α stabilization. Sorafenib is a pan-VEGF receptor inhibitor and thus by its anti-angiogenic action can increase tumor hypoxia. Increased hypoxia after sorafenib treatment promotes immunosuppression [237, 238]. Immunosuppression

in HCC after sorafenib treatment is promoted by increasing tumor hypoxia through VEGF inhibition. This leads to increased intratumoral expression of the immune checkpoint inhibitor PD-L1 and accumulation of T-reg cells and M2-type macrophages. Hypoxia-induced upregulation of stromal cell-derived (SCD)-1 alpha mediates recruitment of these cells; hence, inhibition of SCD-1 alpha receptor (CXCR4) may prevent the immunosuppression. Though anti-PD-L1 antibody has anti-tumor potential, its potency is enhanced with inhibition of CXCR4. Hence, anti-VEGF therapy combined with immunotherapy may be a potential therapy for HCC [237, 238]. The augmentation of anti-tumor effect of peptide vaccine by increasing immune response of vaccine-induced CTLs via PD-1/PD-L1 blockade further supports the need to develop GPC3 peptide vaccine and α PD-1 antibody combination therapy [239] (Fig. 4).

Hormonal therapy

Males have two times higher risk of developing fibrosis and HCC compared to females. Among females, risk increases with age and post-menopausal women have higher risk of developing fibrosis and HCC than premenopausal age. The difference in the severity of fibrosis and HCC with gender and age indicate the probable role of sex hormone in the disease pathogenesis and may be due to the protective effects of estrogen against fibrogenesis and inhibition of the stellate cell activation with estrogen. Menopausal state in women with decreased estrogen level is associated with mitochondrial dysfunction, cellular senescence, decreased immune response to injury, and increased oxidative stress [240–242]. Collectively, these changes may increase the susceptibility to liver fibrosis and HCC. Since decreased estrogen is associated with increased susceptibility to HCC, estrogen therapy might be beneficial in decreasing the progression of disease. The protective effect of estrogen through IL-6 restrictions, STAT3 inactivation, tumor-associated macrophage inhibition, and decreased inflammation has been documented in patients with HCV and HBV. Furthermore, decreased probability of liver fibrosis has been shown with hormone replacement therapy in post-menopausal women [240–242]. This suggests that estrogen therapy might be used to decrease the progression of disease in the early disease stage. But, diethylnitrosamine-induced development of liver cancer has also been documented. Thus, the effect of estrogen therapy on estrogen receptor, its anti-inflammatory effect, interactions between microRNAs and estrogen in HCC, extent of benefits and overall safety, and the dose and time of estrogen administration warrant further investigations [240–242].

Conclusion

HCC represents more than 90% of the primary liver cancers and is an emerging cause of mortality. The inadequacy of present-day treatment modalities like surgical resection, chemotherapy, radiotherapy and local ablation such as RAF and TACE raises concern and indicates the need for more effective treatments. Host immune response suppression results in the unopposed development of HCC; thus, activation of the host immune system may serve as a potential treatment strategy yielding improved outcomes. Combination therapy, gene therapy, and immunotherapy have shown promising results in treating the tumor directly and in preventing recurrence and reactivation. Immunotherapy of HCC by targeting AFP, Glypican-3, DCs and NK cells, NY-ESO-1, MAGE-A family, SSX-1, and ROBO1 are still in the pre-clinical or clinical trial phase and require further studies to develop effective therapeutic immunotherapy for HCC. Immune checkpoint inhibition and adoptive cell transfer therapy are other treatment modalities in the developing phase. The existing therapies for the treatment of HCC have shown promising results. However, immunogenicity and the presence of an immunological barrier, viral toxicity, side effects from chemotherapy, and refractoriness to chemotherapy, drug resistance, and problems in translating the research findings into clinical utility remain major concerns.

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Compliance with ethical standards

Conflict of interest The authors have no other relevant affiliations or financial involvement with any organization or entity with the financial interest or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed. No writing assistance was utilized in the production of this manuscript.

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