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Chapter 4

Immunotherapy for Hepatocellular Carcinoma: Current Status and Future Perspectives

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Additional information is available at the end of the chapter

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1. Introduction

For most patients with advanced hepatocellular carcinoma (HCC), surgery with curative intent or a locally ablative technique, such as percutaneous ethanol injection or radiofrequency ablation, are no longer available [1]. Patients can now be treated using transarterial chemoembolization (TACE) or systemic chemotherapy. Several chemotherapeutic drugs have been developed and tested. The anti-tumor effect of these treatments is limited and adverse reactions are not tolerated in advanced HCC patients with liver cirrhosis, which affects drug metabolism and toxicity [1-3]. Thus far, sorafenib, a multi-targeted tyrosine kinase inhibitor, is the only drug that has been shown to significantly prolong survival (by nearly 3 months) in patients with advanced HCC [4, 5]. However, the incidence of adverse drug reactions is high, particularly in elderly patients, and no second-line treatment has been established for patients who have failed sorafenib treatment [6]. Thus, new treatment modalities are urgently required to prolong survival in patients with advanced HCC while minimizing the risk of adverse reactions.

The 5-year recurrence rate of HCC exceeds 70% after surgery or radiofrequency ablation due to a high risk of metastasis and development of *de novo* HCC in a cirrhotic liver [7,8]. The relapse-free survival rate was reported to be improved by adjuvant therapy with vitamin K2 [9], retinoid [10], or interferon [11-13]. These reports have not as yet been validated, and these treatments to prevent relapse are not widely adopted. In recent years, clinical trials of sorafenib have been conducted to explore its role in adjuvant therapy [14]. However, these data are unpublished and a standard adjuvant therapy has not been established. Establishment of an effective preventative method, such as vaccination to prevent the occurrence and recurrence of HCC, is also required.

Immunotherapy is a potentially attractive option for HCC, and induction of tumor-specific reactions without autoimmunity is the ideal strategy. Many fundamental studies have demonstrated that tumor cells can be targeted by various immune effector mechanisms. Previous immunotherapeutic clinical trials in patients with advanced HCC have shown mainly its feasibility and safety [15,16]. However, no non-randomized phase I or II studies have demonstrated the efficacy of immunotherapy for advanced HCC [16]. Conversely, several randomized controlled trials, in adjuvant settings, have shown its ability to reduce the risk of cancer recurrence [17-19].

This chapter aims to overview current knowledge concerning the progress of immunotherapy for HCC, including preclinical data and clinical trials, and to introduce our fundamental studies and clinical trials of the glypican-3 (GPC3)-derived peptide vaccine.

2. Concepts of antitumor immunity

The aim of immunotherapy against cancer is to provide clinical benefit by activating the immune system. Various immunotherapy strategies have been investigated in preclinical and clinical trials to accomplish this purpose. The diversity of strategies is due to the fact that tumor cells can be targeted by various immune effector mechanisms, such as lymphokine-activated killer (LAK) cells, natural killer (NK) cells, T cells, dendritic cells, cytokine therapy, and antibody treatment. The induction of long-lasting tumor-specific reactions without autoimmunity is the ideal immunotherapeutic strategy and has been investigated extensively, particularly for melanoma and renal cell carcinoma. Rosenberg reported a dramatic clinical effect of adoptive cell therapy (ACT) using autologous tumor-infiltrating lymphocytes (TILs) against metastatic melanoma [20]. Also, TILs derived from HCC, after *ex vivo* expansion with interleukin-2 (IL-2), can lyse autologous tumors [21]. Furthermore, patients with HCC infiltrated by lymphocytes demonstrate a better prognosis after resection [22]. Thus the immune system, activated in various ways, can recognize and eliminate cancer cells, including HCC, although these cells may develop various mechanisms of escape from this action (Figure 1).

2.1. HCC antigenic targets

Tumor-specific antigens are the principal targets of immunotherapy, including in cancer vaccines, in ACT, and as monoclonal antibodies (mAbs). Thus, identification of appropriate tumor-specific antigens is the first and important step for progress of immunotherapy. Tumor-specific CD8+ T cells are considered to be critical for cancer control. They recognize 8- to 11-amino acid peptides that are derived from intracellular proteins called tumor antigens, which are presented in association with HLA class I complexes. Various tumor antigens and their cytotoxic T lymphocyte (CTL) epitopes have been identified and investigated in HCC.

Alpha-fetoprotein (AFP) is a representative HCC tumor-specific antigen. The onco-fetal antigen AFP, considered an ideal serological marker, is expressed in 50–80% of HCC. Various human leukocyte antigen (HLA)-A2- or HLA-A24-restricted AFP-specific epitopes have been

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identified. AFP has been shown to be an effective tumor rejection antigen in murine HCC [23]. Additionally, an AFP-derived peptide vaccine has been demonstrated to induce antigen-specific CD8 T-cell response in HCC patients [24]. In HCC, AFP is the most commonly investigated antigen, and several AFP-based immunotherapy regimens have been reported; however, no dramatic clinical benefit was observed [24,25].

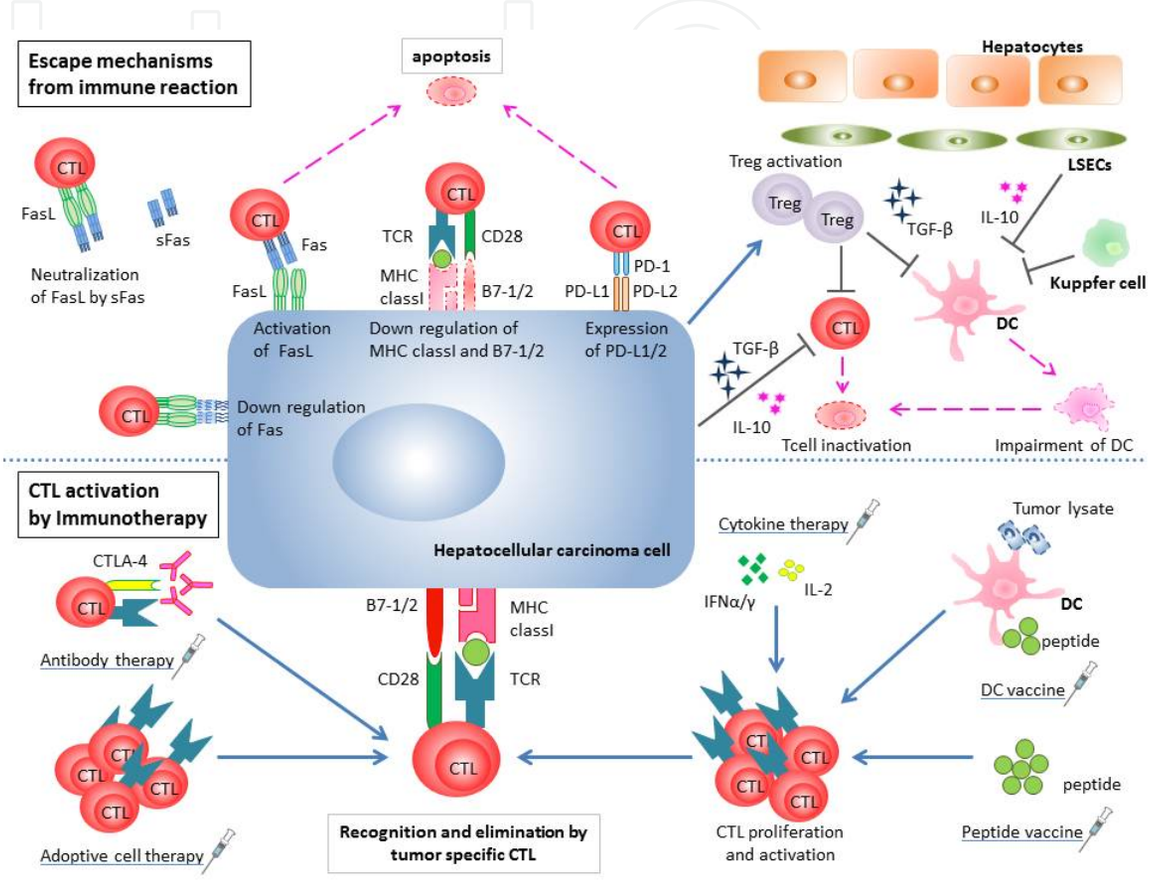

Figure 1. Immunotherapy against hepatocellular carcinoma cells. A number of strategies exist for induction of antitumor immunity against hepatocellular carcinoma cells. Tumor-specific cytotoxic T lymphocytes (CTLs) activated by various immunotherapies are capable of recognizing and eliminating cancer cells. However, tumor cells have developed various mechanisms of escape from antitumor reactions. Increased comprehension of the mechanisms underlying the immune-privileged status of the liver and escape of tumors from immune reactions will increase the efficacy of immunotherapy.

MAGE and NY-ESO-1, cancer testis antigens, are also expressed in HCC tumors. Normally, tumor testis antigens are expressed only in the testis and/or ovary. Additionally, major histocompatibility complex (MHC) class I antigens are not expressed on germ cells; thus, they are considered promising cancer vaccine candidate antigens. MAGE-A was initially identified in melanoma [26], and later found to be expressed in another cancers, including HCC [27], lung cancer, breast cancer, oral squamous cell carcinoma, and esophageal carcinoma. Some CTL epitopes of the MAGE family have been identified in HCC.

NY-ESO-1 was identified in a patient with squamous cell carcinoma of the esophagus [28]. NY-ESO-1 is expressed in various cancers, including melanoma, lung cancer, ovarian cancer,

breast cancer, and HCC. NY-ESO-1 is characterized by its high immunogenicity and is considered a good target molecule for antigen-specific immunotherapy.

GPC3, a heparan sulfate proteoglycan, was previously reported to be overexpressed in HCC [29]. The carcinoembryonic antigen GPC3 plays an important role in cell growth and differentiation and is considered an ideal tumor antigen for immunotherapy; this antigen is discussed further below.

2.2. Dendritic cells

Dendritic cells (DCs) are the most potent antigen-presenting cells (APCs), and are composed of multiple subsets, primarily conventional and plasmacytoid DCs [30]. DCs play an important role in both induction of antitumor immunity and tolerance. The DC vaccine, loaded with tumor-specific antigens, is considered to stimulate a specific T-cell response. Several methods of antigen loading to DCs exist, including peptide pulsing, whole protein loading, and genetic engineering. DC-based immunotherapy is highly complex due to the various possible strategies, such as the DC subset used, the method of antigen loading, and the administration route (subcutaneous, intravenous, intralymph node, or intratumoral). Figdor *et al*. provided a roadmap for standardization and quality control of DC vaccines [31]. In HCC patients, enhanced NK-cell activation and decreased regulatory T-cell (Treg) frequencies have been identified after administration of DC vaccines [32]. Many studies suggested that DC-based immunotherapies for HCC could stimulate a tumor-specific T-cell response leading to clinical benefit without any significant toxicity.

2.3. Cytokine therapy and immunostimulatory mAbs

The effects of immunostimulatory cytokines in HCC have been investigated, such as interferon-alpha (IFN-α), interferon-gamma (IFN-γ), and interleukin (IL)-2. These elicit a nonspecific immune response.

As an antiviral agent, IFN-α is often used against hepatitis B or hepatitis C virus infection to prevent progression to HCC. IFN-α, by enhancing cytotoxicity, tumor antigen presentation, proliferation of lymphocytes, and anti-angiogenesis, induces an antitumor response [33,34]. IFN-α treatment for HCC has been reported to have some clinical efficacy, likely by preventing or delaying tumor recurrence after surgical resection or ablation [35,36]. IFN-α has been tested in combination with chemotherapy for advanced HCC [37,38]. Adverse side effects are an important issue in IFN-based therapy, particularly for patients with severe liver injury.

IFN-γ, which improves antigen presentation and lymphocyte activation, has also been used for advanced HCC in combination with chemotherapy [39] or granulocyte-macrophage colony stimulating factor (GM-CSF) [40]. However, no clinical response was identified.

IL-2, one of the most immunostimulatory cytokines, plays an important role in regulation of immune activation and homeostasis. IL-2 has various effects on immune cells, such as CD4+ T cells, CD8+ T cells, B cells and NK cells [41]. The effect of IL-2 in various cancers has been

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investigated, particularly melanoma and renal cell carcinoma. In HCC, several IL-2 treatment regimens have been reported, with or without combination therapy [42,43].

In 1975, the procedure for generation of hybridomas was published [44]. Subsequently, mAbs have been developed as diagnostic and therapeutic agents. In the field of cancer therapy, mAbs that activate the immune system against tumor cells, inhibit cancer cell-intrinsic signaling pathways, bring toxins close to cancer cells, or interfere with the tumor-stroma interaction have been developed [45].

Several anti-costimulatory molecule antibodies that activate the immune response have been investigated. For example, a mAb against the costimulatory molecule CD28, the receptor of the family of B7 antigens, has been investigated. For T-cell activation, both binding of the T-cell receptor to antigen and costimulatory signaling by CD28 are needed [46]. Some CD28 mAbs called 'superagonists' can stimulate and expand T cells in the absence of T cell antigen receptor (TCR) ligation [47]. In a phase I trial of an anti-CD28 mAb, severe toxicity was observed [48].

The CTL-associated antigen 4 (CTLA-4), a homolog of CD28, is an inhibitory receptor for B7 [49] that functions as an immune check point and downregulates T-cell activation pathways by competing with CD28 for binding to B7 [50,51]. The clinical benefit of ipilimumab, anti CTLA-4 mAb, against advanced melanoma has been reported [52,53]; its use has been approved by the United States Food and Drug Administration.

2.4. Escape mechanisms from immune reactions

As mentioned above, cancer cells can be targeted by various immunotherapeutic strategies. However, cancer cells possess mechanisms of escape from the immune response. Additionally, the liver is considered an immune-privileged organ. The liver contains at least three types of APCs; *i.e.*, Kupffer cells (KCs), liver sinusoidal endothelial cells (LSECs), and dendritic cells, which might be associated with its immune-privileged status [54]. KCs and LSECs constitutively express the anti-inflammatory cytokines IL-10 and transforming growth factor beta (TGF-β) [55,56]. These immunosuppressive cytokines may play a role in immune privilege by influencing T-cell differentiation and suppressing APC maturation. Furthermore, hepatic stellate cells (also known as Ito cells), a liver-specific cell population that is found between the sinusoids and hepatocytes, promote hepatic inflammation. Hepatic stellate cells express TGF-β only after chronic liver injury [57,58].

2.4.1. Impairment of DC function

One of the mechanisms of tumor escape from the immune response is impairment of DC function. In cancer patients, inadequate DC function has been suggested to be related to non-responsiveness to antitumor immunity [59]. Immunosuppressive factors that inhibit DC maturation are released from tumors. For instance, human cancer cells release vascular endothelial growth factor (VEGF), which inhibits the maturation of DCs [60]. Other cytokines derived from tumors, such as IL-6 [61] and IL-10 [62], also influence the function of DCs.

Additionally, DCs have reduced function in cancers, including HCC, in that they cannot stimulate T cells [63,64].

2.4.2. Antigen presentation

It is clear that the level of MHC class I expression on the cell surface is crucial for CD8+ T cell cytotoxicity against target cells. Decreased or absent MHC class I expression, which facilitates tumor escape from immune surveillance, has been reported in various tumors. Additionally, in HCC, HLA class I expression on tumor cells may be down-regulated [65,66]. However, strong HLA class I expression in HCC has also been reported [67]. Thus, the level of MHC class I expression in HCC is unclear. Furthermore, expression of the co-stimulatory molecules B7-1 and B7-2 is reduced in HCC [66]. Such down-regulation causes impairment of tumor-antigen processing and presentation.

2.4.3. Inhibitory molecules

Another escape mechanism involves over- or reduced expression of molecules associated with cell death, such as Fas/FasL, PD-1/PD-L1, CTLA-4, and Decoy receptor 3. Fas is a cell-surface protein that belongs to the family of tumor necrosis factor (TNF) receptors [68]. Fas ligand (FasL) is a type II membrane protein that binds to Fas [69]. Cross-linking of Fas with FasL induces apoptosis of Fas-bearing cells [70]. FasL is found in immune-privileged sites, such as the testis and eye [71,72]. HCC tissues have been reported to express Fas weakly and at a low frequency [73]. Additionally, elevated soluble Fas (sFas) levels in HCC patients have been reported [74]. Loss of cell-surface Fas in HCC and neutralization of FasL by sFas might be involved in tumor cell immune escape [75].

PD-L1 is member of the B7 family that can interact with programmed death-1 (PD-1). Its receptor, PD-1, is expressed on activated T and B cells and elicits inhibitory signals [76]. PD-L1 is expressed on dendritic cells, macrophages, and parenchymal cells, as well as various human cancer cells. The objective response of the PD-1 antibody against non-small cell lung cancer, melanoma, or renal cell cancer has been suggested to be related to PD-L1 expression on tumor cells [77]. In HCC, PD-1 expression is upregulated on effector-phase CD8+ T cells, particularly in tumor-infiltrating CD8+ T cells [78]. High expression of PD-1 on T cells both in TILs and peripheral blood mononuclear cells (PBMCs) is correlated with a poor prognosis in HCC patients after surgical resection [78]. Additionally, PD-L1 expression on Kupffer cells (KC) has been shown to be increased in tumor tissues in patients with HCC, and is correlated with poor survival [79]. These suggest that effector phase T-cell inhibition is associated with tumor survival.

Decoy receptor 3 (DcR3), a member of the TNF receptor superfamily, might also be involved in immune escape. DcR3 inhibits FasL-induced apoptosis by binding to its ligand Fas. Additionally, DcR3 overexpression in HCC has been reported [80,81].

2.4.4. Regulatory T cells

CD4+CD25+ regulatory T cells (Tregs) can suppress other immune cells and are critical mediators of self-tolerance. Tregs also suppress the immune response against cancer cells.

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High numbers of Tregs were detected in peripheral blood and TILs in HCC patients [82, 83]. CD4+CD25+FoxP3+ Tregs could impair the cytotoxic function of tumor-infiltrating CD8+ T cells [84]. Levels of the immunosuppressive cytokine IL-10 are increased in HCC patients, a finding that is related to Treg induction [85]. Thus, CD4+CD25+ Tregs may play an important role in regulating the immune response against HCC.

The goal of immunotherapy against human cancers, including HCC, is to impact target tumor cells without influencing normal cell function. Comprehension of the mechanisms of the immune-privileged status of the liver and escape of tumors from immune reactions will increase the efficacy of immunotherapy.

3. Clinical trials

Clinical trials of immunotherapy to enhance anti-tumor responses in patients with advanced HCC, or to reduce the risk of recurrence after curative treatment have been conducted (Table 1).

Author	Country	Year	Indication	Immunotherapy	n	Clinical result	Reference
Takayama T, et al.	Japan	2000	Adjuvant (resection)	RCT: activated autologous lymphocyte vs. no treatment	76 and 74	Significantly longer recurrence-free survival after transfer of activated lymphocytes (p=0.008)	[17]
Llovet JM, et al.	Spain	2000	Advanced HCC	RCT: IFN-α2b vs. no treatment	30 and 28	RR: 2/30 (7%), DCR: NA No significant difference in RR and survival	[156]
Ikeda K, et al.	Japan	2000	Adjuvant (resection or ethanol injection)	RCT: IFN-β vs. no treatment	10 and 10	Significantly longer recurrence-free survival after IFN-β therapy (p=0.0004	[11]
Kubo S, et al.	Japan	2001	Adjuvant (resection)	RCT: IFN-α vs. no treatment	15 and 15	Significantly longer recurrence-free survival after IFN-α therapy (p=0.037)	[12]
Reinisch W, et al.	Austria	2002	Advanced HCC	GM-CSF + IFN-γ	15	RR: 1/15 (7%), DCR: 10/15 (67%) MST: 5.5 months	[40]
Palmieri G, et al.	Italy	2002	Advanced HCC	Low dose IL-2	18	RR: 3/18 (17%), DCR: 16/18 (89%) MST: 24.5 months	[42]

Author	Country	Year	Indication	Immunotherapy	n	Clinical result	Reference
Ladhams A, et al.	Australia	2002	Advanced HCC	Dendritic cell pulsed with autologous tumor	2	Slowing in the rate of tumor growth in one of two patients	[157]
Sakon M, et al.	Japan	2002	Advanced HCC	5-FU + IFN-α	11	RR: 8/11 (73%), DCR: 9/11 (82%) MST: NA	[158]
Iwashita, et al.	Japan	2003	Advanced HCC	Dendritic cell pulsed with autologous tumor	10 (8 HCC)	RR: 0/8 (0%), DCR 6/8 (75%) MST: NA	[114]
Patt YZ, et al.	USA	2003	Advanced HCC	5-FU + IFN-α2b	43	RR: 9/36 (25%), DCR 22/36 (61%) MST: 19.5 months	[37]
Stift A, et al.	Austria	2003	Advanced HCC	Dendritic cell pulsed with autologous tumor	20 (2 HCC)	RR: NA, DCR: NA MST: 10.5 months Constant remaining of AFP over a period of 6 months in one of two patients	[159]
Feun LG, et al.	USA	2003	Advanced HCC	Doxorubicin + 5-FU + IFN-α2b	30	RR: 2/30 (7%), DCR: 3/30 (10%) MST: 3 months	[160]
Komorizono Y, et al.	Japan	2003	Advanced HCC	Cisplatin + 5-FU + IFN-α	6	RR: 2/6 (33%), DCR 3/6 (50%) MST: NA	[38]
Butterfield, et al.	USA	2003	Advanced HCC	AFP peptide vaccination	6	RR: 0/6 (0%), DCR 0/6 (0%) MST: 8 months	[24]
Shiratori Y, et al.	Japan	2003	adjuvant (ethanol injection)	RCT: IFN-α vs. no treatment	49 and 25	Longer recurrence-free and overall survival after IFN-α therapy (p-value not shown)	[13]
Kuang M, et al.	China	2004	Adjuvant	RCT: autologous formalin-fixed tumor vaccine vs. no treatment	18 and 21	Significantly longer recurrence-free survival after vaccination (p=0.003)	[18]
Shi M, et al	China	2004	Advanced and early HCC	Cytokine induced killer cell	13	RR: NA, DCR: NA MST: NA	[161]
Sangro B, et al.	Spain	2004	Advanced HCC	Intratumoral adenovirus encoding IL-12 genes	21 (8 HCC)	RR: 1/8 (13%), DCR 7/8 (88%) MST: NA	[162]

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Author	Country	Year	Indication	Immunotherapy	n	Clinical result	Reference
Lee WC, et al.	Taiwan	2005	Advanced HCC	Dendritic cell pulsed with autologous tumor	31	RR: 4/31 (13%), DCR 21/31 (68%) MST: NA	[163]
Kumagai, et al.	Japan	2005	Advanced HCC	Intratumoral dendritic cell injection after ethanol injection	4	Feasibility study	[164]
Yin XY, et al.	China	2005	Advanced HCC	Cisplatin + doxorubicin + 5-FU + IFN-2α	26	RR: 4/26 (15%), DCR 13/26 (50%) MST: 6 months	[165]
Chi KH	Taiwan	2005	Advanced HCC	Local radiation + intratumoral DC injection	14	RR: 2/14 (14%), DCR 9/14 (64%) MST: 5.6 months	[113]
Mazzolini G, et al.	Spain	2005	Advanced HCC	Dendritic cell transfected with adenovirus encoding IL-12 gene	17 (8 HCC)	RR: 0/0 (0%), DCR: 2/8 (25%) MST: NA	[166]
Butterfield, et al.	USA	2006	Advanced HCC	Dendritic cell pulsed with AFP peptide	10	RR: 0/10 (0%), DCR 0/10 (0%) MST: 7.5 months	[25]
Nakamoto Y, et al.	Japan	2007	Advanced and early HCC	Non-RCT: TACE + dendritic cell vs. TACE alone	10 and 11	No significant difference in survival	[141]
Vitale FV, et al.	Italy	2007	Advanced HCC	5-FU + IFN-α2b	9	RR: 3/9 (33%), DCR 4/9 (44%) MST: 11.5 months	[167]
Weng DS, et al.	China	2008	Adjuvant (TACE and RFA)	RCT: cytokine induced killer cell vs. no treatment	45 and 40	Significantly longer recurrence-free survival after immunotherapy (p=0.01)	[168]
Hui D, et al.	China	2009	Adjuvant (resection)	RCT: cytokine induced killer cell 3 courses vs. 6 courses vs. no treatment	41, 43 and 43	Significantly longer recurrence-free survival after immunotherapy (p=0.001 and 0.004)	[169]
Palmer DH, et al.	UK	2009	Advanced HCC	Dendritic cell pulsed with liver tumor cell line lysate (HepG2)	35	RR: 1/25 (4%), DCR 7/25 (28%) MST: 5.6 months	[170]
Olioso P, et al.	Italy	2009	Advanced HCC	Cytokine induced killer cell + IFN-α	12 (1 HCC)	Complete response Survival time: 33 months (alive)	[171]

Author	Country	Year	Indication	Immunotherapy	n	Clinical result	Reference
Hao MZ, et al.	China	2010	Advanced HCC	Non-RCT: TACE + cytokine induced killer cell vs. TACE alone	72 and 74	Significantly longer survival after combination therapy (p<0.001)	[172]
Greten TF, et al	Germany	2010	Advanced HCC	a telomerase peptide vaccine in combinatuon with a low dose cyclophosphamide	40	RR: 0/40 (0%), DCR 17/37 (45.9%) MST: 9.8 months	[139]
Ma H, et al.	China	2010	Adjuvant (RFA)	RFA and autologous RetroNectin activated killer cells	7	During a seven-month follow-up, no severe adverse events, recurrences or deaths	[173]
Zhou P, et al.	China	2011	HCC with hepatitis B(PMWA)	Immature DCs, cytokine-induced killer cells (CIK), cytotoxic T lymphocytes (CTL) and tumor lysate-pulsed DC	10	This phase I study revealed this therapy was safe and increased the percentage of effector cells.	[174]
Sawada Y, et al.	Japan	2012	Advanced HCC	GPC3-derived peptide vaccine	33	RR: 1/33 (3%), DCR 20/33 (60.6%) MST: 9.0 months OS was significantly longer in patients with high GPC3-specific CTL frequencies	[120]

HCC; hepatocellular carcinoma, LAK; lymphokine-activated killer cell, IL; interleukin, RR; response rate, DCR; disease control rate, MST; median survival time, IFN; interferon, NA; not assessed, RCT; randomised control trial, CTL; cytotoxic T lymphocyte, TIL; tumor-infiltrating lymphocyte, TACE; transcatheter arterial chemoembolization, GM-CSF; granulocyte macrophage colony-stimulating factor, RFA; radiofrequency ablation therapy, PMWA; percutaneous microwave ablation

Table 1. Immunotherapeutic clinical trials in HCC after 2000

3.1. Cytokine therapy

3.1.1. IFN-α

IFN-α has direct antitumor effects on tumor cells, including induction of lymphocytes, macrophage cytotoxic activities, and anti-angiogenesis.

A number of trials have evaluated the clinical efficacy of IFN-α in HCC. Lai *et al*. reported that IFN-α was useful in patients with inoperable HCC, in terms of both prolonging survival and inducing tumor regression [86]. However, a high IFN-α dose can cause toxicity [12]; thus, systemic administration of IFN-α [12] or IFN-β [11] should be considered

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as supportive treatment after hepatectomy or tumor ablation, which may prevent or delay tumor recurrence. Combination therapy with IFN-α and chemotherapy was applied in advanced HCC patients; however, no benefit was identified other than tolerance of the therapy for cirrhotic patients [13].

3.1.2. IL-2

IL-2 is an immunostimulatory cytokine that is used singly or in combination with other treatments in patients with liver tumors. Systemic induction of IL-2 produces objective responses against HCC when administered alone [42] or in combination with melatonin [43] or lymphokine-activated killer (LAK) cells [87].

3.1.3. IFN-γ

Lygidakis *et al*. reported that combination therapy with hepatic transarterial locoregional chemotherapy and immunotherapy that included IFN-γ and IL-2 is a promising therapeutic approach for advanced HCC [39]. This highlights the effect of IFN-γ. Moreover, GM-CSF and IFN-γ were effective in selected advanced HCC patients [40].

Systemic IL-12 and TNF-α treatment has been reported to cause severe toxicity in other cancers. However, there is to our knowledge no report of their effect against primary or metastatic liver cancer.

Although cytokine treatment for HCC can have positive outcomes, toxic effects can result, including systemic vascular leak syndrome.

Cytokines, such as IL-7 and IL-15, may be reasonable adjuvants due to their vaccination and culture properties.

3.2. Gene transfer

Transfer of immunostimulatory cytokine genes has effects on immune tolerance against tumors. Clinical trials with gene transfer therapy have been evaluated. Presently, this procedure is a safe and represents a novel therapeutic approach.

There are two main approaches to transfer of genes: 1) direct injection of vectors expressing cytokines, chemokines, or costimulatory molecules into tumor lesions, or 2) use of tumor cells or DCs transduced *ex vivo* with vectors expressing cytokines or costimulatory molecules [88].

IL-12 is a potent cytokine that shows antitumor activity in some models [89,90]. Although the effect of IL-12 gene transfer for liver tumor treatment in animal models has been reported, its use in early clinical trials of cancer patients has shown no significant benefit [91].

Abnormally elevated levels of Th2 cytokines, such as IL-10, skews the immune response to favor tumor growth. Conversely, Lopez *et al*. showed that the combination of autologous inactivated tumor cells expressing IL-12 and IL-10 induced tumor remission in 50–70% of mice with large established colon or mammary tumors and spontaneous lung metastases, with consequent establishment of an antitumor immune memory [92]. Systemic injection of IL-2 in

patients with metastatic renal carcinoma and melanoma showed a low efficacy and high toxicity. A phase I–II clinical trial of recombinant adenovirus encoding the IL-2 gene was performed in patients with advanced carcinoma. Only one patient showed a positive response in terms of tumor necrosis [93].

Molecules such as HLA-B7 are important for promotion of specific T-cell responses. Total or selective loss of MHS class I antigens has been reported in some malignancies [94,95]. Animal studies have demonstrated that injection of foreign MHC molecules can result in immunologic destruction of the tumor by eliciting a T-cell-dependent immune response not only to the foreign MHC protein, but also to previously unrecognized tumor-associated antigens. Rubin *et al*. showed that indirect intralesional gene transfer therapy of both HLA-B7 and β2-microglobulin for colorectal cancer (CRC) patients with hepatic metastasis had no serious toxicity and was feasible; however, details of any antitumor effect were not reported [96].

Oncolytic virotherapy is based on the ability of viral vectors to replicate selectively in cancer cells and thus exert a direct antitumor effect [97]. Adenovirus is one of the most common viral vectors [98]. dl1520 is a mutant oncolytic adenovirus [99]. Habib *et al*. reported that dl1520 gene therapy had no significant antitumor effect in HCC patients compared with percutaneous ethanol injection [100]. A phase I clinical trial of intratumoral administration of a first-generation adenoviral vector-encoding herpes simplex virus thymidine kinase (HSV-TK) gene (Ad.TK) to HCC patients was conducted. Treatment was well-tolerated and no dose-limiting toxicity occurred. Sixty percent of patients showed tumor stabilization and, importantly, two patients who received the highest dose showed signs of intratumoral necrosis using imaging procedures [101].

Additionally, Kottke *et al*. showed that, in mice, oncolytic virotherapy could lead to direct tumor cell lysis and could trigger innate immune-mediated attack on tumor vascularization when combined with antiangiogenic cancer therapy [102].

Transfer of cytokine genes and oncolytic viruses is currently under development and represents a promising new approach for treatment of human cancer. Recent technical advances in the genetic modification of oncolytic viruses have improved their tumor specificity. Clinical trials with oncolytic viruses demonstrate the safety and feasibility of this approach. Systemic administration of oncolytic viruses represents a novel approach to treatment of a range of tumors [103].

3.3. Effector cells and adoptive T-cell therapy

Several trials have evaluated the induction of various types of cytotoxic lymphocytes. One report compared adoptive chemoimmunotherapy with chemotherapy. Chemoimmunotherapy comprised arterial infusion of adriamycin, recombinant interleukin-2, and lymphokine-activated killer cells, whereas chemotherapy comprised administration of adriamycin alone. No significant difference between the two groups was found; thus adoptive chemoimmunotherapy was concluded to not be an ideal adjuvant protocol after hepatic resection [104].

The reason that LAK cells demonstrate no benefit may be their lack of tumor-antigen specificity. In contrast, TILs with anti-tumor activity are induced during the natural course of tumor

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growth. Thus, TILs have been shown to contain tumor antigen-specific T cells [20]. In one study, indium^{111}-labeled TILs activated by IL-2 and CD3 mAbs were injected via intrahepatic arteries in three patients with hepatic malignancies and their distribution was evaluated. TILs accumulated in the liver and persisted for at least 48 h after infusion. After intra-arterial chemoimmunotherapy that included TILs, two of three patients achieved a partial therapeutic response. This method may facilitate accumulation of TILs at tumor sites, likely augmenting the antitumor effects of adoptive immunotherapy [105].

In the largest randomized trial, 150 patients who had undergone curative resection for HCC received either IL-2 with anti-CD3-activated peripheral blood lymphocytes or underwent observation. Adoptive immunotherapy decreased the frequency of recurrence and prolonged the time to first recurrence compared with the control group. Additionally, the immunotherapy group demonstrated a significantly longer recurrence-free survival and disease-specific survival than the control group. However, overall survival did not differ significantly between groups, providing more objective support for the potential of immunotherapy [17].

Adoptive T-cell therapy includes passive transfer of antigen-reactive T cells to a tumor-bearing host to initiate tumor rejection. Based on animal models, effector T cells with tumor-specific reactivity are superior to non-specific effector T cells in terms of mediating tumor regression *in vivo* [106].

However, translation of these successful methods into patients is not yet feasible due to difficulties in generation of tumor antigen-specific T cells *ex vivo* [107]. In general, adoptive T-cell therapy is accomplished by harvesting cells from peripheral blood, tumor sites (TILs), or draining lymph nodes, and identifying tumor-associated antigens (TAAs). TAAs are ectopically expressed or overexpressed in tumor cells relative to normal tissues. One of the most important HCC TAAs is AFP. AFP-based immunotherapy has been applied in HCC. Grimm *et al*. immunized mice bearing m-AFP-expressing HCC using DNA expression vectors encoding mAFP. Some mice developed mAFP antibody responses, which were associated with a significant survival benefit. These data suggested that AFP has the potential to function as a tumor antigen, inducing CTLs and CD4+ T-cell-mediated regression of AFP-positive HCC [108].

Many other TAAs that are tumor-specific "cancer-testis" antigens in HCC (MAGE, GAGE, BAGE, NY-ESO, CTA, TSPY, FATE/Bj-HCC-2, and GPC3, among others) have been identified [109]. GPC3 is a specific immunomarker of HCC and induces effective antitumor immunity in mice [110]. Several antigens, such as CEA and CP1, are also known to be TAAs of CRC liver tumors [111].

3.4. APCs

A number of strategies utilize the immune-activating ability of professional APCs, particularly DCs. T-cell activation can result from DC cross presentation. Thus, mature DCs can induce antitumor immunity [112]. A phase I study of the safety and efficacy of direct injection of autologous immature DCs into tumors under radiotherapy was conduct-

ed. A decrease in the AFP level of greater than 50% was identified in three patients, and NK activity was enhanced [113].

Addition of tumor lysate or purified proteins to immature DCs improves their function as APC. Iwashita *et al*. used autologous DCs pulsed with tumor lysate (TL) and evaluated their safety and feasibility. Immunization with TL-pulsed DCs was well-tolerated and feasible. In one patient, one of two liver tumors showed necrotic changes and, in two patients, serum levels of tumor markers decreased after vaccination [114].

Morse *et al*. concluded that combination therapy with DCs pulsed with a CEA peptide and adjuvant cytokines (IFN-α and TNF-α) in patients with CEA-expressing malignancy showed no toxicity and was feasible [115]. Brat *et al*. showed that peptide-loaded DCs enhanced NK cell activation and decreased Treg frequencies in vaccinated HCC patients [32].

Thus, the potential of DCs to improve treatment of many cancers has been confirmed, and various strategies are now being developed.

3.5. Peptide vaccines

Douglas *et al*. showed that gp100 peptide vaccine and IL-2 combination therapy resulted in progression-free survival longer than IL-2 alone in patients with advanced melanoma [116]. The peptide vaccine was tolerated and yielded favorable immunologic responses, such as induction of peptide-specific CTLs or reduced Tregs [117,118].

Regarding HCC, the AFP-derived peptide vaccine induced antigen-specific CD8 T-cell responses; however, no dramatic clinical benefit was identified [24].

The GPC3-derived peptide vaccine can induce high-avidity CTLs capable of killing GPC3-expressing HCC cells [119]. A phase I trial of the GPC3-derived vaccine for advanced HCC indicated that the vaccine was well-tolerated and that peptide-specific CTLs could be a predictive marker of overall survival [120]. The GPC3 peptide vaccine is discussed further in the next section.

4. The GPC3-derived peptide vaccine: our fundamental sutdies and clinical trials

4.1. GPC3, an ideal tumor antigen

GPC3 is a member of the glypican family of heparan sulfate proteoglycans, which are attached to the cell surface via the glycosylphosphatidylinositol (GPI) anchor [121]. GPC3 forms a complex with Wnt molecules and promotes the growth of HCC by stimulating canonical Wnt signaling [122]. We reported that GPC3 was specifically overexpressed in human HCC based on cDNA microarray data [29]. We reported that GPC3 is an ideal tumor antigen for immunotherapy in mouse models [110] and is correlated with a poor prognosis in human HCC [123,124]. We identified both HLA-A24*(A*2402)* and H-2K^{d}-restricted GPC3$_{298–306}$ (EYIL-

SLEEL), as well as HLA-A2*(A*0201)*-restricted $GPC3_{144-152}$ (FVGEFFTDV), as peptides that can stimulate GPC3-reactive CTLs without inducing autoimmunity [110,125]. By performing a binding assay, we confirmed that the HLA-A*02:01-restricted $GPC3_{144–152}$ (FVGEFFTDV) peptide can also bind to HLA-A*02:06 and HLA-A*02:07. We also conducted a preclinical study in mice to design an optimal schedule for a clinical trial of the GPC3-derived peptide vaccine. This preclinical study showed that incomplete Freund's adjuvant (IFA) is indispensable for peptide-based immunotherapy, and that the immunological effect of the peptide vaccine was dose dependent [126].

4.2. Phase I clinical trial of a GPC3-derived peptide vaccine

Based on these results, we conducted a phase I clinical trial of this GPC3-derived peptide vaccine in patients with advanced HCC, the results of which were published recently [120]. Thirty-three advanced HCC patients were administered GPC3 vaccination intradermally (injections on days 1, 15, and 29 with dose escalation). $GPC3_{298-306}$ (EYILSLEEL) was used in HLA-A24-positive patients and $GPC3_{144-152}$ (FVGEFFTDV) in HLA-A2-positive patients. GPC3 peptide vaccination was well tolerated. One patient showed a partial response, and 19 showed stable disease 2 months after initiation of treatment. Four of the 19 patients with stable disease had tumor necrosis or regression that did not meet the criteria for a partial response. The disease control rate (partial response + stable disease) was 60.6%, 2 months after initiation of treatment. Levels of the tumor markers AFP and/or des-γ-carboxy prothrombin temporarily decreased in nine patients. We also analyzed the GPC3-specific CTL frequency by *ex vivo* IFN-γ enzyme-linked immunospot (ELISPOT) assay. In 30 patients, numbers of GPC3 peptide-specific CTLs increased in peripheral blood after GPC3 peptide vaccination. We established several $GPC3_{144–152}$ peptide-specific CTL clones with antigen-specific killing activity against tumor cells from PBMCs of patients vaccinated in this trial [119]. Tumor biopsies were performed (with informed consent) in seven patients to evaluate infiltration of CD8-positive T cells by immunohistochemical staining. Many CD8-positive T cells infiltrated tumors after vaccination. This study showed that the peptide-specific CTL frequency was correlated with overall survival in HCC patients receiving peptide vaccination. In multivariate analysis, the GPC3 peptide-specific CTL frequency was the predictive factor for overall survival in this trial. Analysis of all 33 patients showed that the median overall survival was 12.2 months (95% confidence interval, 6.5 to 18.0) in patients with high GPC3-specfic CTL frequencies, compared with 8.5 months (95% confidence interval, 3.7 to 13.1) in those with low GPC3-specfic CTL frequencies ($P = 0.033$). This study provided much immunological evidence that suggested the potential for improvement of overall survival.

4.3. Ongoing trials of GPC3-based immunotherapy

We subsequently conducted a phase II study of the GPC3-derived peptide vaccine as an adjuvant therapy for patients with HCC (University Hospital Medical Information Network Clinical Trials Registry, UMIN-CTR number: 000002614). Forty patients with initial HCC who had undergone surgery or radiofrequency ablation were enrolled in this phase II, open-label, single-arm trial. Ten vaccinations were performed over 1 year after curative treatment. The

primary endpoints were the 1- and 2-year recurrence rates. The secondary endpoints were immunological responses, as measured by IFN-γ ELISPOT assay. Currently, the correlation between the time of recurrence and immunological responses is being analyzed.

We are conducting a subsequent trial for advanced HCC to assess whether TILs with an anti-tumor effect are indeed increased (UMIN-CTR number: 000005093). In all cases, liver biopsies will be performed before and after GPC3 peptide vaccination, according to the protocol. In the phase I trial, we did not confirm whether the TILs detected after vaccination were GPC3 peptide-specific. In the ongoing trial, we could detect GPC3 peptide-specific CTLs in liver biopsy specimens by flow cytometry using dextramer staining.

We expect that the results of these studies will validate the biomarkers and provide a rationale for a larger randomized clinical trial to determine the efficacy of the GPC3-derived peptide vaccine. Conversely, the antitumor effect in advanced cancer of the peptide vaccine alone is not dramatic. Thus, we aim to develop combinatorial approaches [127] or strong antigen-specific immunotherapies, such as ACT following lymphodepletion [20]. Additionally, clinical trials of the adoptive transfer of GPC3-specific CTLs in patients with HCC in Japan are planned [128].

5. Development of immunotherapy and potential of combined therapy

Combinatorial strategies could comprise either a combination of classic chemo- or radiotherapy or simultaneous application of different immunotherapeutic approaches. Many preclinical studies have shown synergistic effects of combined therapy, standard cytotoxic chemotherapy [127], or radiotherapy [129]. Elimination or inhibition of Treg activity by low-dose cyclophosphamide or antibodies against CD25 was shown to be a rational approach [130-132]. Simultaneous administration of antibodies against CTLA-4 [133] or PD-1[131] may modify the tumor immunosuppressive microenvironment, thereby increasing the efficacy of immunotherapy.

5.1. Potential of combination therapies

Some chemotherapeutic agents upregulate TAA expression or reduce tumor cell resistance to specific CTLs [134]. Subtoxic-dose chemotherapy increased the susceptibility of tumor cells to the cytotoxic effect of CTLs [127].

Cell-surface expression of MHC class I molecules was increased for many days in a radiation dose-dependent manner using a murine model [135]. Conversely, exposing HCC to low-dose radiation increases the efficacy of DC-mediated immunotherapy due to upregulation of MHC class II and Fas expression after irradiation [136].

HCC thermal ablation induced or enhanced T-cell responses specific for HCC–associated antigens in PBMCs derived from 20 patients with HCC [137]. Similarly, the effect on the immune system of radiofrequency ablation was greater than that of surgical resection in both HCC patients and tumor-bearing mice. All seven patients with GPC3-expressing HCCs

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exhibited an increase in GPC3-specific CTLs after radiofrequency ablation or TACE, but not after surgical resection [138].

5.2. Clinical trials of combinatorial approaches

Several clinical trials of combinational approaches have been reported.

Greten *et al*. reported the effect of low-dose cyclophosphamide treatment in combination with telomerase peptide (GV1001) vaccination in 40 patients with advanced HCC [139]. GV1001 treatment resulted in a decrease in the number of CD4+CD25+Foxp3+ Tregs; however, no GV1001-specific immune responses were detected after vaccination.

Conversely, a randomized phase II trial of a multiple tumor-associated peptide vaccine for renal cell carcinoma showed that a single dose of cyclophosphamide reduced the number of Tregs and that immune responders had prolonged survival if pretreated with cyclophosphamide (hazard ratio = 0.38; $P = 0.040$) [140]. There was no difference in survival of nonimmune responders in the cyclophosphamide and non-cyclophosphamide arms. Thus the synergistic effects of cyclophosphamide might require a specific immune response.

Nakamoto *et al*. reported that transcatheter arterial DC infusion into tumor tissues following transarterial embolization treatment was feasible and safe in 10 patients with cirrhosis and HCC [141]. There was a trend for patients infused with DCs to display a longer recurrence-free survival. Thus transcatheter arterial infusion might be rational for specifically inducing immune effects in the target lesion.

Thus far, few clinical trials of the combination of immunotherapy and chemotherapy in HCC have been reported because chemotherapy, with the exception of sorafenib therapy, has not been demonstrated to be useful. Further studies are necessary to increase the clinical efficacy of immunotherapy for advanced HCC. There is hope that the combination of well-designed clinical trials of innovative immunotherapeutic approaches will lead to development of efficient new therapies for treatment of HCC.

5.3. mAbs

Use of mAbs that target tumor antigens is an important therapeutic approach for cancer treatment. mAbs can act as both agonists and antagonists by binding important key receptors to control immune responses [142].

5.3.1. CD28

Antibodies against CD28 are known to induce antitumor immunity in combination with bi-specific antibodies that bind to both the tumor antigen and the TCR-CD3 complex [143]. However, CD28 antibodies can activate T cells directly, as shown in a phase I dose escalation trial using a CD28 mAb that reported severe toxicity, including a systemic inflammatory response. Thus infusion of CD28 mAbs is associated with serious difficulties [48].

5.3.2. CD137

CD137, a member of the TNF receptor superfamily, is expressed on antigen-activated T cells (CD4+,Cd8+ Tregs and NK cells), DCs, cytokine-activated NK cells, eosinophils, mast cells, and endothelial cells of some metastatic tumors, and binds to a high-affinity ligand expressed on several APCs such as macrophages and activated B cells [144]. An anti-CD137 mAb promoted survival of T cells and prevented cell death [145,146]. These suggest that anti-CD137 mAbs can enhance T cell-mediated immune responses. Melero *et al*. reported the antitumor effect of an anti-CD137 mAb on Ag104 sarcoma and P815 mastocytoma in mice [144].

Unfortunately, Niu *et al*. reported that single injection of anti-CD137 caused anomalies such as splenomegaly, hepatomegaly, lymphadenopathy, multifocal hepatitis, anaemia, altered trafficking of B cells and CD8+ T cells, loss of NK cells, and a 10-fold increase in bone marrow cells bearing the phenotype of hematopoietic stem cells [147].

5.3.3. OX40

OX40 (also known as CD134 and TNR4) is a member of the TNFR family that is expressed on activated CD4+ and CD8+ T cells. The OX40 ligand is expressed on activated APCs (DC, B cells, and macrophages), and possibly also on activated T cells and endothelial cells. OX40 ligand stimulates T-cell proliferation and ensures T-cell long-term survival. OX40 or OX40L deficiency leads to weaker CD4+ T-helper immune responses in mice. Moreover, expression of exogenous OX40L by tumor cells increases their immunogenicity, and causes their rejection by CD4+ T helper 1 cells and CTL responses. No side effects induced by OX40 ligand have yet been reported, although the possibility cannot be excluded because OX40 has been found on CD4+ lymphocytes infiltrating multiple sclerosis and inflammatory bowel disease lesions. Phase I clinical trials of a murine anti-human OX40 mAb have been initiated in patients with advanced cancer of multiple tissue origins, although repeat administration of this xenogeneic antibody will be limited due to immune responses against the murine sequences of the antibody [148].

5.3.4. GPC3

Chugai Pharmaceutical Co., Ltd. developed the GPC3 antibody (GC33) for treatment of HCC. They demonstrated antitumor efficacy of GC33 in several human liver cancer xenograft models and the important role of antibody-dependent cellular cytotoxicity (ADCC) in the antitumor mechanism of GC33. They also showed that macrophages play an important role in this antitumor activity, which is unlikely to be direct ADCC by macrophages themselves [149]. Clinical trials of GC33 in advanced HCC patients are ongoing.

5.3.5. CTLA-4

CTLA-4 is an immunosuppressive receptor on T cells. Via ligand binding, CTLA-4 generates inhibitory signals that reduce T-cell proliferation and IL-2 secretion. Administration of CTLA-4 mAbs demonstrated antitumor effects in some murine malignant models [150,151].

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Prieto *et al*. followed patients with melanoma treated with CTLA-4 mAb (ipilimumab) and either the gp100 peptide or IL-2. Ipilimumab induced durable, potentially curative tumor regression in a small percentage of patients with metastatic melanoma; furthermore, combination with IL-2 increased the complete response rate [152]. Some phase II clinical trials have reported the safety and therapeutic effect of CTLA-4 mAb in HCC patients. CTLA-4 mAb showed promising antitumor effects against HCC in addition to antiviral activity against hepatitis C virus [153].

5.3.6. PD-1

PD-L1 is a member of the B7 family that can interact with programmed death-1 (PD-1). Its receptor PD-1 is expressed on activated T and B cells and elicits inhibitory signals [76]. A phase I trial using a fully human IgG4 PD-1 blocking antibody (MDX-1106) demonstrated objective responses with limited toxicity in patients with treatment-refractory solid tumors [154]. The objective responses of non-small cell lung cancer, melanoma, or renal-cell cancer associated with PD-1 antibody may be related to PD-L1 expression on tumor cells [77]. In HCC, PD-L1 expression is correlated with tumor aggressiveness and postoperative recurrence [155].

A number of other mAbs have demonstrated benefits for the treatment of HCC as well as undesired effects associated with their high affinity and selectivity. The most promising observations are that mAb therapies have synergistic effects in combination with other strategies.

6. Conclusion

To date, there is no report of adequate antitumor efficacy of immunotherapy in clinical trials involving advanced HCC patients. However, the available data suggest that immunotherapy has the potential to improve survival without impairing the quality of life, and is expected to be effective for prevention of recurrence.

Immunotherapy for HCC is still in the preclinical and clinical trial phases of development; however, it will become available and be clinically successful in the near future. Analysis of the correlation between clinical and immunological responses is required for to demonstrate the efficacy of immunotherapy. The challenge remains to design clinical trials to appropriately evaluate novel immunotherapies or combination therapies, and allow feedback to facilitate ongoing development.

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