Review

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Status of and prospects for cancer vaccines against hepatocellular carcinoma in clinical trials

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Summary **Current therapies to treat advanced hepatocellular carcinoma (HCC) are not satisfactory because of the high rate of recurrence after treatment and because of severe complications after surgery. Cancer vaccines have been studied for decades to achieve effective, micro-invasive, long-lasting anti-tumor action. Cancer vaccines are designed to promote tumor-specific immune responses and increase specific cytotoxic CD8-positive T cells. This review summarizes 16 phase I clinical trials of cancer vaccines against HCC that have been conducted over the past 10 years. According to those trials, the Alpha fetoprotein (AFP), Glypican-3 (GPC3), and Multidrug resistance-associated protein 3 (MRP3) vaccines were well tolerated and safe. Some early clinical trials have shown that vaccination resulted in a large number of T cells activated by a specific tumor-associated antigen in the circulation, but clinical outcomes were not satisfactory. This may be because targets for immunosuppressive agents have yet to be clearly determined in HCC. Therapeutic regimens that combine activative agents and suppressive agents may profoundly improve clinical outcomes for patients with HCC in the future.**

Keywords: Hepatocellular carcinoma (HCC), cancer vaccine, tumor-associated antigen

1. Introduction

Hepatocellular carcinoma (HCC) is one of the most common cancers, with a global incidence of over 600,000 new cases per year. In 2012, HCC caused about 746,000 deaths worldwide (*1*). Although early diagnosis and treatment improve survival, HCC is rarely cured, and it frequently recurs. Hepatic resection can improve the 5-year recurrence-free survival rate by up to 25% (*2-6*). The 5-year survival rate after surgery was 57% for patients with resected lesions <5cm and 32% for patients with tumors > 10cm. Median survival after recurrence is about 7-28 months (*4*). Molecular techniques have detected micro metastases of HCC in 88% of patients at the time of surgery (*7*), and these metastases probably cause HCC to recur postoperatively.

Local regional therapy is palliative and its modalities include microwave ablation, radiofrequency ablation (RFA), transarterial embolization (TAE), and percutaneous alcohol embolization. The median overall survival for patients with unresectable HCC after local regional therapy is less than 1 year (*8*). HCC is notoriously resistant to chemotherapy and other systemic treatment modalities. The multi-targeted kinase inhibitor sorafenib improves survival by 2.3-2.8 months and is the only systemic drug that has been found to increase survival time in patients with advanced HCC (*9*). However, sorafenib is quite expensive.

Immunotherapy to treat HCC has been studied for decades (*10*) and appears quite promising in light of recent advances in the treatment of other malignancies such as melanoma. Unlike melanoma, however, HCC produces characteristic findings in the liver since

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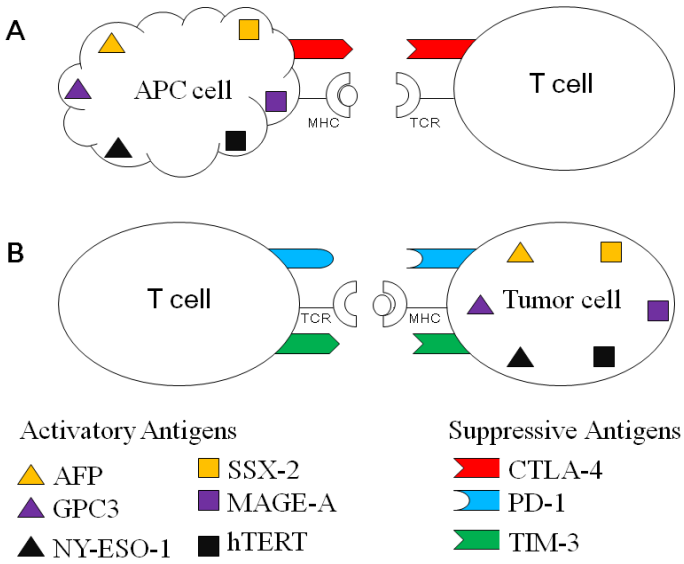

Figure 1. Mechanism of the immune response that kills HCC cells. Three main stepwise events must be initiated and allowed to proceed and progress iteratively. 1, Neoantigens created by oncogenesis are released and captured by APCs for processing. 2, DCs present the captured antigens on MHC-I and MHC-II molecules to T cells, resulting in the activation of effector T cell responses to cancer-specific antigens. 3, Cytotoxic lymphocytes specifically recognize and bind to cancer cells through interaction between T cell receptors (TCR) and their cognate antigens bound to MHC-I, and lymphocytes kill their target cancer cell. Immuno-activative agents (*e.g.* cancer vaccines) trigger the activation of cytotoxic T cells in step 1. Immuno-suppressive agents induce cancer cell evasion of cytotoxic lymphocytes in step 2 and step 3 by binding to an antigen (ligand).

most patients with HCC are first infected with the hepatitis B/hepatitis C virus (HBV/HCV). Patients present with unique anti- or pro-tumor responses during the development and progression of HCC (*11*). The adaptive immune system, including type 1 helper T cells (Th1 cells), cytotoxic T lymphocytes (CTLs), and dendritic cells (DCs), is weakened during interaction with a chronic HBV/HCV infection. Regulatory T cells (Tregs) and myeloid-derived suppressor cells (MDSCs) enhance or suppress the immune reaction. Excessive activation of immunosuppressive cells can contribute to persistent infection with HBV/HCV and the progression of HCC (*12*).

Immunotherapy to treat HCC can be categorized into several types depending on the strategy. For instance, immune modulators and tumor vaccines are used to enhance the immune response to HCC in an indirect way, while adoptive immunotherapy introduces a large amount of immune cells in a direct way. The current review summarizes and analyzes 16 phase I clinical trials (Figure 1) to illustrate the status of and prospects for cancer vaccines against HCC.

2. Immuno-activative agents targeting HCC

Activative immunotherapy is provided with tumor associated antigens. The specific immuno-reaction induced by tumor-associated antigens (TAAs) is a key requirement for success of T cell immunotherapy. There are now 7 major TAAs that have been intensively studied to treat HCC as shown in Table 1.

2.1. *a-Fetoprotein (AFP)*

Alpha fetoprotein (AFP) is an oncofetal antigen over-expressed by many HCCs. AFP is a tumor marker for HCC and it can also be used as a potential target for immunotherapy. AFP is over-expressed in most HCCs and thus offers an attractive target for immunotherapy against this neoplasm. Several studies (*13,14*) have showed that anti-HCC effects were achieved in a therapeutic setting with a DNA vaccine encoding mouse AFP and co-expressing the heat shock protein 70 (HSP70) gene. This vaccine elicited a marked and highly effective AFP-specific CTL response to AFP-positive target cells. This vaccine also prolonged the lifespan of tumor-bearing mice and it eliminated HCC. Four major AFP epitopes, hAFP137-145, hAFP158-166, hAFP325-334, and hAFP542-550, have previously been reported (*15*). In patients with HCC, the large proportion of AFP-specific $CD8^+$ T cells directed against different epitopes suggests that AFP has strong and broad immunogenicity (*15,16*). Recently, a study (*14*) examined two patients with advanced HCC who received a vaccination with three plasmid DNA injections followed by a single AdV injection after surgery. Results of that study showed the vaccine was well tolerated and safe. After injection of the AFP vaccine, the patients' AFP levels remained within the normal range. The level of AFP-specific $CD8^+$ T cells remained high. Both patients showed immunologic evidence of immunization, but the clinical results were not satisfactory. The first patient developed AFP-expressing HCC again after nine months. The second

Table 1. Clinical trials of immuno-activative agents targeting HCC

Target tumor antigen	Frequency of antigen presentation	$CD8^+$ T cell response	Presentation of HLA	References
AFP	70.4%	6 /6	A0201	2001 (*15*)
		6 /6	A0201	2006 (*16*)
		24 /40	A0201	2008 (*44*)
		2/2	A0201	2014 (*14*)
GPC3	74.5%	11 /22	A2, A24	2006 (*19*)
		30/33	A2, A24	2012 (*20*)
NY-ESO-1	27.8%	5 /6	A2	2004 (*21*)
		10 /28	A2	2004 (*45*)
		6 /10	A2	2009 (*24*)
		18/96	A2	2014 (*22*)
SSX- 2	37.5%	3 /6	A2	2005 (*23*)
		3 /10	A2	2009 (*24*)
MAGE-A	16.7%	1 /6	A1, A2	2004 (*25*)
TERT	36.4%	3 /6	A2	2005 (*23*)
		5/16	A24	2006 (*26*)
MRP3	72.7%	9/11	A24	2015 (*27*)

In 15 clinical trials of cancer vaccines against HCC, the AFP, GPC3, and MRP3 vaccines had a specific T cell response rate of over 70%. GPC3, NY-ESO-1, SSX- 2, MAGE-A, and TERT had a T cell response rate below 40%.

patient developed HCC again after 18 months without an increase in serum AFP. The lack of impressive clinical outcomes in prior studies is presumably why no randomized controlled trials of AFP have been conducted until now.

2.2. *Glypican-3 (GPC3)*

Glypican-3 (GPC3) is highly expressed in HCC cell lines such as HepG2, Hep3B, HT17, HuH6, HuH7, and PLC/PRF/5 (*17,18*). Expression of GPC3 protein is found in more than 70% of HCCs but not in normal liver tissue when using a rabbit polyclonal antibody raised against human GPC3 (*17*). Transgenic mice have been used to identify HLA-A2-restricted GPC3 epitopes in order to expand the use of GPC3-based immunotherapy to patients with HLA-A2+ HCC (*19*). The GPC3 (144-152 residues) peptide can induce peptide-reactive CTLs in transgenic mice. A phase I clinical trial of a GPC3-derived peptide vaccine for advanced HCC has identified HLA-A24-restricted GPC3 144-152 and HLA-A2-restricted GPC3 298-306 CTL epitope peptides in HCC cells and it has shown that GPC3-reactive CTLs can be generated from PBMCs stimulated with these peptides in about 50% of patients with HCC (*20*). The trial in question was a nonrandomized, open-label, phase I clinical trial in patients with advanced HCC. The clinical response, however, was not satisfactory. Of 33 patients, only one had a partial response (PR) and 19 had a stable disease (SD) for 2 months. The median time to tumor progression (TTP) was 3.4 months and the median overall survival was 9.0 months.

2.3. *NY-ESO-1*

NY-ESO-1 is a family of cancer-testis antigens, the expression of which is limited to tumor tissue and the testes. Expression of NY-ESO-1 mRNA has been detected in 20-30% of cancers. NY-ESO-1 mRNA has also been detected in about 30% of HCCs according to reverse transcription-PCR. HCC can be naturally immunogenic with NY-ESO-1 as a tumor-specific antigen that elicits both humeral and cellular responses in patients with HCC (*21*). A study has found specific $CD8^+$ T-cell responses to NY-ESO-1b in 48% of patients with NY-ESO-1 $mRNA^+HLA\text{-}A2^+$ HCC, suggesting the potential for use of NY-ESO-1 in immunotherapy for patients with NY-ESO-1 $mRNA^+HLA\text{-}A2^+$ HCC (*22*). No studies have examined the clinical response of patients immunized with NY-ESO-1 vaccines.

2.4. *SSX- 2*

SSX-2 is another cancer-testis antigen. Analysis of its expression with Northern blotting and RT-PCR revealed the presence of SSX-2 transcripts in a significant proportion of human cancers, such as melanomas (35%), head and neck cancers (35%), lymphomas (36%), and colon carcinomas (12%). A study found that SSX-2-specific T cells are spontaneously activated *in vivo*, extending natural immunogenicity to HCC (*23*). However, some clinical trials have noted a low level of T cells specific for SSX-2 (*23,24*)

2.5. *Melanoma antigen-encoding gene A (MAGE-A)*

The melanoma antigen-encoding genes (MAGE A1-A6) are a family of cancer-testis antigens. These genes are silent in normal cells, with the exception of male germline cells that do not express HLA class I and are therefore unable to present antigens to CTL. For these reasons, these genes are of particular interest in cancer immunotherapy. A study has found that MAGE/tetramer+ CD8 cells in tumor tissue from patients with HCC are able to recognize the MAGE-1 sequence 161-169 and the MAGE-3 sequence 271-279, making this antigen a potential candidate for a MAGE-specific immunotherapy to treat HCC (*25*). However, specific T cells were induced in only 1 of 6 patients in a clinical trial.

2.6. *Human telomerase reverse transcriptase (hTERT)*

Human telomerase reverse transcriptase (hTERT) has been identified as the catalytic enzyme required for telomere elongation. Recently, several findings regarding the hTERT-specific cytotoxic T cell (CTL) responses in humans and mice have been reported (*26*). CTLs stimulated with peptides or cell lines with DNA-based immunization have high levels of hTERT, suggesting that hTERT-reactive T cell clones are not deleted from the human T cell repertoire and that hTERT may be a useful tumor-specific antigen as a target for T-cell-based immunotherapy for cancers. However, the existence of hTERT-specific CTLs and the relationship between immunological responses and clinical factors have not been studied intensively in patients with HCC.

2.7. *Multidrug resistance-associated protein 3 (MRP3)*

Multidrug resistance-associated protein 3 (MRP3) is a member of the family of ATP-binding cassette (ABC) transporters expressed on the cell surface. MRP3 is expressed at high levels in various cancer cells. A study (*27*) showed that vaccination induced MRP3-specific immunity in 72.7% of patients. However, the median overall survival time for 12 patients in a non-controlled trial was 14.0 months. MRP3 did not provide a marked clinical benefit.

Overall, trials have been conducted to examine the clinical response to the AFP, GPC3, and MRP3 vaccines, but the clinical outcomes were not satisfactory. However, these vaccines did induce a larger number of specific T cells than did other vaccines. There are no studies of the clinical response to NY-ESO-1, SSX- 2, MAGE-A, or TERT vaccines.

3. Immuno-suppressive agents targeting HCC

Using the immune system to cure the cancer is just like driving a car. Triggering immuno-activity is just like pressing the gas pedal. If the brake is still on, the car cannot move. Immuno-suppression acts just like the brake system. For an immune response to effectively kill HCC cells, a series of stepwise events must be initiated and allowed to proceed and progress iteratively (Figure 1) (*28*). In the first step, neoantigens created by oncogenesis are released and captured by antigen-presenting cells (APCs) for processing. In the second step, dendritic cells (DCs) present the captured antigens on MHC-I and MHC-II molecules to T cells, resulting in the activation of effector T cell responses to the cancer-specific antigens. In the third step, cytotoxic lymphocytes specifically recognize and bind to cancer cells through interaction between T cell receptors (TCR) and their cognate antigens bound to MHC-I, and lymphocytes kill their target cancer cells. Immuno-activative agents trigger specific T cell proliferation in the first step. Immuno-suppressive agents induce cancer cells to evade cytotoxic lymphocytes of the immune system in the second or third steps by binding to their antigens (ligands). The immuno-suppressive agents receiving the most attention are summarized below.

3.1. *Programmed death 1 (PD-1)*

Programmed death 1 (PD-1), a co-inhibitory receptor molecule, is induced on activated T and B cells and plays a crucial role in regulating peripheral tolerance (*30*). The ligand for PD-1, PD-L1 (also known as B7-H1), is expressed on DCs and tumor cells. Substantial evidence has demonstrated that PD-L1 can deliver an inhibitory signal to PD-1-expressing T cells, leading to suppression of the immune response by induction of apoptosis, anergy, unresponsiveness, and functional exhaustion of T cells.

Anti-PD-1 and anti-PD L1 therapeutics have greatly evolved over the past year. Pembrolizumab (previously known as lambrolizumab) was the first anti-PD-1 antibody to obtain FDA approval (September 2014). It was approved as a second-line treatment for unresectable stage III or stage IV metastatic melanoma. Nivolumab was the second anti-PD-1 antibody to obtain FDA approval (December 2014) (*31*).

Why can anti-PD-1 not be used to treat patients with HCC? A study (*32*) found that the prevalence of circulating $PD\text{-}1^{+}CD8^{+}$ T cells increased with disease progression from liver cirrhosis to HCC in patients in comparison to healthy controls. However, $PD\text{-}1^{+}CD8^{+}$ T cells were found in fewer than 40% of patients with HCC (*22/50*). Therefore, these findings have increased our knowledge of the role of the PD-1/PD-L1 pathway in tumor evasion, but the PD-1/PD-L1 pathway is not the major reason for immune evasion by HCC.

3.2. *Cytotoxic T lymphocyte-associated antigen 4 (CTLA-4)*

Cytotoxic T lymphocyte-associated antigen 4 (CTLA-

4) is a T cell trans-membrane receptor. Binding CTLA-4 to its natural ligands down regulates T-cell activation. In humans, a CTLA-4 blockade with anti-CTLA-4 mAbs inhibits downregulation of the immune system, enhancing and prolonging T cell activation and producing durable antitumor responses (*33*). A CTLA-4 blockade is one of the first molecularly targeted approaches to immunotherapy and constitutes a novel and promising strategy to control cancer. Intensive research into immunotherapy has made substantial progress, particularly as clinical trials have confirmed the efficacy of T cell stimulation in patients suffering from malignant melanoma (*34*) and renal cancer (*35*). At present, two different anti-CTLA4 mAbs are being clinically developed as cancer therapeutics. Tremelimumab, or CP-675,206 (Pfizer, Inc., New London, Connecticut, USA), and ipilimumab, or MDX-010 (Bristol-Myers Squibb/Medarex, Princeton, New Jersey, USA), have demonstrated antitumor activity alone and in combination with other agents in patients with advanced melanoma (36).

Despite the promising results obtained thus far, clinical trials of CTLA-4 inhibitors in HCC are limited. A CTLA-4 blockade combined with microwave ablation and local GM-CSF administration displayed moderate efficacy in subcutaneous models of HCC (*37*). A phase I trial of tremelimumab in patients with HCC has recently been reported (NCT01008358) (*38*). The trial enrolled 21 patients with chronic hepatitis C with Child-Pugh A or B cirrhosis and advanced HCC not amenable to percutaneous ablation or transarterial embolization. Tremelimumab was well tolerated, and there were no treatment-related deaths. Almost half of the patients experienced a grade 3/4 rise in AST but this was not associated with a parallel decline in liver dysfunction. Partial responses were seen in 17.6% of patients, and 45% of patients had SD for about 6 months. The median time to progression was 6.48 months and the median overall survival was 8.2 months. Although the non-controlled design of this trial had its limitation, its findings do warrant further study.

3.3. *T cell immunoglobulin mucin 3 (TIM-3)*

The T cell immunoglobulin mucin (TIM) family are considered to be critical checkpoint proteins in the regulation of multiple phases of the immune response and in maintaining immune system homeostasis (*39*). Increased Tim-3 expression on $CD4^+$ and $CD8^+$ T cells has been noted in patients with chronic hepatitis B (*40*). Up-regulation of Tim-3 expression in natural killer (NK) cells by HBV infection has also been found to suppress the functioning of NK cells in patients with chronic hepatitis B (*34,41*). Moreover, over-expression of Tim-3 has been implicated in T cell dysfunction and exhaustion that occur in cancers. Therefore, Tim-3 is an important negative regulatory receptor that potentially contributes to T cell dysfunction and exhaustion in both chronic HBV infection and cancers.

4. Prospects for research on cancer vaccines against HCC

Numerous studies have found that cancer vaccines against HCC lack satisfactory clinical efficacy. Despite this, cancer vaccines are still being tested as a potential therapy, and particularly in combination therapies, in clinical trials in order to improve outcomes. Therapeutic vaccines are an option in place of marginal resection, local ablation treatments such as percutaneous alcohol injection and radio-frequency thermal ablation, or trans-arterial chemo-embolization. One study administered a vaccine after surgery (*42*) while another administered immunotherapy after microwave ablation (*43*). These clinical studies have found those approaches to be effective to an extent.

The TAAs that have a potential to treat HCC are AFP, GCP-3, and MRP3 because these vaccines are well tolerated and safe. The rate of a specific T cell response was over 70% for these vaccines. In contrast, the T cell response rate for GPC3, NY-ESO-1, SSX- 2, MAGE-A, and TERT was below 40%. Thus, the first three TAAs need to be studied further as cancer vaccines in order to improve clinical outcomes for patients with HCC. Immuno-activative and immunosuppressive agents should be used in combination. Although anti-PD1 has recently made breakthroughs in the treatment of melanoma, renal cancer, and lung cancer, an anti-PD1 regimen has not proven efficacious in treating HCC, as was mentioned earlier. Accordingly, other immunosuppressive agents should be examined in the same way as many TAAs were examined. In the future, clinical studies should examine cancer vaccines combining activative agents and suppressive agents. AFP, GPC-3, and MRP3 should be studied as TAAs for use in cancer vaccines.

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