Engineering α -fetoprotein-based gene vaccines to prevent and treat hepatocellular carcinoma: review and future prospects

Activation of a patient's immune system offers an attractive approach to prevent and treat hepatocellular carcinoma (HCC). However, the antitumor efficacy of current HCC vaccines was weak owing to insufficient immune activation of targeting self/tumor antigens. We recently found that epitope-optimized α -fetoprotein effectively activated CD8 T cells and generated potent antitumor effects in the carcinogen-induced autochthonous HCC mouse model. We predict that the same antigen engineering approach of epitope-optimization will enable us to develop effective human vaccines to prevent HCC recurrence after liver resection. The engineered human HCC vaccines may also allow us to identify high-affinity T-cell receptors and antibodies that can be used to reprogram T cells to treat HCC tumors via adoptive transfer.

Keywords: antigen engineering • cancer vaccine • epitope optimization • genetic immunization • hepatocellular carcinoma • immunotherapy • recombinant lentivector

Background

Liver cancer, with approximately 750,000 new cases and approximately 695,000 deaths annually, is the fifth most common cancer and the third most common cause of cancer death in the world [1]. According to the CDC, the incidence rate of hepatocellular carcinoma (HCC), the most common type of liver cancer, is increasing significantly in the USA, while many other major cancers are declining [2]. The number of new HCC cases in the USA has almost tripled over the past two decades to reach an estimated 24,500 in 2013 (American Cancer Society). The hepatitis B virus (HBV) vaccines have markedly reduced new HBV infections and should decrease the number of HBV-associated HCC cases in the long run [3]. However, the large pool of existing chronic HBV (350 million) [4] and hepatitis C virus (HCV; 170 million) [5]-infected patients, and the recent recognition that obesity/diabetes has become a major contributing factor of HCC in the USA [6,7], suggests that HCC incidence in the USA and worldwide may continue increasing for the next few decades. Unfortunately, the treatment options

for HCC are limited and ineffective [8], which contribute to the dismal 3-year survival rate of 17% [1]. Although liver resection and transplantation are potentially curative for small, early-stage HCC tumors, the lack of postsurgery adjuvant therapies is a critical barrier to the success of HCC surgical treatment. For example, the 5-year recurrence rate of HCC following liver resection is approximately 70% [9]. Thus, there is an urgent demand to develop novel approaches to HCC treatments that can prevent relapse after surgery and HCC *de novo* development in high-risk populations such as HBV and HCV chronically infected patients. One of the approaches is to enlist a patient's own immune system to prevent HCC *de novo* development and to guard against HCC recurrence [10,11]. Multiple immunological approaches involving both cellular and humoral arms of the immune system have been explored as potential immunotherapy approaches for treating HCC in mouse models and human clinical trials (see **Figure 1**) [12–14]. In this review, we first focus our efforts on reviewing previous literature reports of HCC immunotherapy

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Figure 1. Immunological approaches used in hepatocellular carcinoma immunotherapy at the stage of *in vitro* study in animal models or in clinical trials.

AFP: α -fetoprotein; Ag: Antigen; CIK: Cytokine-induced killer cell; DC: Dendritic cell; GPC3: Glypican-3; HCC: Hepatocellular carcinoma; HSP: Heat shock protein.

based on the cellular immune responses; secondarily, we propose and discuss antigen engineering strategies to create more effective HCC vaccines. For antibody-based HCC immunotherapy, readers are encouraged to examine an excellent recent review article by Feng and Ho [15].

Current cellular immune approaches for HCC immunotherapy

A number of approaches have been exploited to activate the cellular arm of the host immune system to treat liver cancer in both animal models and human trials. These approaches are either antigen-specific or -nonspecific and can be grouped into passive immunotherapy (adoptive immunotherapy) and active immunotherapy (cancer vaccines).

Passive immunotherapy

The passive immunotherapy approach involves the transfer of *in vitro*-activated (antigen-specific or -non-specific) autologous immune effector cells to cancer patients in order to mediate tumor killing *in vivo*. Thus, it is also referred to as adoptive immunotherapy or adoptive cell transfer. While the tumor-infiltrating lymphocytes or even antigen-specific T cells are frequently used in the immunotherapy of melanoma, the most widely used immune effector cell in the field of HCC immunotherapy is the cytokine-induced killer (CIK) cells, which include NK, T and NKT cells. The CIK cells are generated *in vitro* from peripheral blood

cells by stimulation with a cocktail of cytokines consisting of IFN- γ , IL-2 and anti-CD3 antibodies. The antigen specificity of CIK cells is undefined (or may be antigen nonspecific). The function of CIK cells may not be restricted by MHC molecules [16,17].

In 2000, Takayama *et al.* reported the results of the first randomized trial of using CIK cells to prevent recurrence of HCC following liver resection [18]. In total, 150 postoperative HCC patients were divided into the CIK cell-treated and control groups. After a median of 4.4 years follow-up, investigators found that CIK cell immunotherapy decreased overall recurrence by 18% (59 vs 77%). The time to recurrence in the CIK cell-treated group was significantly longer than that of the control group ($p = 0.01$). However, the overall survival was not significantly different. In another clinical trial, postoperative HCC patients were followed up for 5–7 years after CIK cell treatment [19]. The authors corroborated Takayama *et al.*'s findings by showing that adoptive transfer of CIK cells could significantly prolong the disease-free survival. Once again, the overall survival rate was not improved by CIK cell treatment. Consistent with these reports, two other clinical trials also demonstrated that adoptive transfer of CIK cells did in fact significantly delay the 1- and 3-year recurrence after surgical treatment of HCC, but did not significantly affect the overall 3-year survival [20,21]. However, in a recent study with a considerably larger group of patients (>400 patients), Pan *et al.* demonstrated that the adoptive transfer of CIK cells

after surgery, especially with eight cycles of CIK cell infusions, significantly improved the overall survival of the patients with HCC tumor size >5 cm [22].

For patients with larger HCC tumors that did not qualify for liver resection, CIK cells have been used in combination with nonsurgical approaches of transcatheter arterial chemoembolization and radiofrequency ablation. In a nonrandomized trial, Huang *et al.* reported that CIK cells increased the overall survival and progression-free survival of HCC patients following transcatheter arterial chemoembolization and radiofrequency ablation treatment [23].

In summary, clinical trials demonstrated that CIK cell adoptive transfer has the potential to delay HCC recurrence following liver resection and to improve the quality of life in advanced HCC patients. However, a major drawback of the CIK cell-mediated immunotherapy is that the antigen-specificity of CIK cells is undefined and the therapy may be nonspecific with a potential for autoimmune disease induction although no side effects have been documented thus far. In addition, the mechanism underpinning the anti-tumor effect of CIK is not well understood. However, because CIK cells are easily prepared and encompass a wide variety of immune effector cells, CIK cells will continue to be a choice of adjuvant therapy for HCC patients. A greater understanding of the mechanism of how CIK cells works may help improve the efficacy of CIK cells in treating HCC. Furthermore, additional stimulation of the CIK cells with autologous dendritic cells (DCs; DC-CIKs) vaccines may generate stronger and antigen-specific cytolytic immune effectors against HCC tumor cells [24].

Active immunotherapy

Active tumor immunotherapy is a means to administer cancer vaccines to activate the host immune system *in vivo* to generate antigen-specific immune responses to achieve an antitumor effect. Based on the antigen spectrum and specificity, HCC cancer vaccines can be divided into two groups: antigen undefined and antigen defined.

Antigen-undefined HCC cancer vaccines

This type of vaccine is based on the same principle as most infectious disease vaccines: the usage of the entire tumor cells or tumor cell lysate as tumor antigens that encompass all potential antigens in the HCC tumor cells. To increase immune responses, autologous DCs are frequently used in the vaccine preparation. In the murine HCC model, immunization with bone marrow-derived DCs, pulsed with mouse Hepa1-6 tumor lysate, could generate a partial therapeutic effect [25]. In a human trial, autologous DCs pulsed with self-HCC

tumor cell lysate were used as vaccines [26]. Among 31 patients, four (12.9%) exhibited partial response, 17 patients' (54.8%) diseases remained stable and ten patients' (32.3%) diseases progressed. The overall 1-year survival rate of all 31 patients was $40.1 \pm 9.1\%$. The patients treated with an additional boosting immunization generated even better 1-year survival rates than those treated with one single immunization ($63.3 \pm 12.0\%$ vs $10.7 \pm 9.4\%$; $p < 0.001$). The DC vaccinations were safe with liver function tests showing no difference before and after immunization. Unfortunately in the study, no immune responses were monitored; thus, it was not possible to correlate the antitumor effect to the vaccine-induced immune activation. In another Phase II clinical trial, lysates from the established allogenic HCC tumor cell line HepG2 were used to pulse autologous DCs [27] for use as cancer vaccines. A total of 35 patients with advanced HCC, who were not suitable for radical or locoregional therapies, received at least three DC vaccinations. The disease control rate (partial response and stable disease ≥ 3 months) determined by imaging was 28%. In 17 patients, the baseline serum α -fetoprotein (AFP) was ≥ 1000 ng/ml; in four of these patients, it fell to $<30\%$ of baseline following vaccination. In one patient, there was a radiological partial response that was associated with a reduction of serum AFP to $<10\%$ of baseline. In addition, the immune responses were assessed in this study by IFN- γ enzyme-linked immunosorbent spot (ELISPOT) assay. In several cases, there was an induction of T-cell responses to the vaccine and/or AFP antigens following vaccinations. Thus, autologous DC vaccination in HCC patients can be considered safe and well tolerated, with evidence of antitumor efficacy and generation of antigen-specific immune responses in some cases. The advantage of using tumor lysate for immunization is the inclusion of a broad spectrum of antigens that should induce a wide range of immune responses against HCC. However, the immune responses activated by such vaccines are mostly weak, generating modest antitumor effect. In addition, the inclusion of all antigens in the hepatocytes has the risk of generating autoimmune hepatitis.

Antigen-defined HCC cancer vaccines

The most promising and exciting HCC immunotherapy approach involves targeting antigens that are specifically expressed or overexpressed in HCC tumor cells. Several HCC-specific tumor-associated antigens have been identified over the last few decades, including oncofetal proteins of AFP [11,12] and glypican (GPC)3 [28], cancer/testis antigens of MAGE-A [29] and NY-ESO [30], and the widely overexpressed antigens such as telomerase reverse transcriptase [31]. HCC

cancer vaccines based on defined HCC-associated antigens have been tested in animal models and clinical trials [11,12,31,32] in the form of peptides, proteins or genes with and without DCs. In this review, we are focusing on AFP- and GPC3-based HCC vaccines owing to their high specificity to HCC.

AFP-based HCC vaccines

AFP is highly re-expressed in 70–80% of HCC [33], and has been used not only as a biomarker for diagnosis, but also as a target for T cell-mediated immunotherapy in animal studies [34,35] and clinical investigations [11,32,36].

Animal studies

In animal models, native mouse AFP plasmid DNA [37–39], DCs transduced with viral vectors [35,40] or pulsed with peptides [41] has been utilized to induce AFP-specific immune responses. However, only low levels of immune activation and antitumor effects have been observed. For example, using the ELISPOT assay, only 10 spots/ 1×10^6 splenocytes were detected after two intramuscular immunizations with mouse AFP plasmid DNA [37]. In another study, three immunizations with bone marrow-derived DCs expressing mouse AFP only protected one out of four mice from AFP-positive tumor challenge [35]. Mouse AFP DNA formulated with cationic polymers generated moderate T-cell responses, although a more pronounced antitumor overall effect was observed [38]. Xenogenic AFP peptides [41] or proteins [42] have also been used to increase AFP-specific immune responses. In addition, a combination of AFP with heat shock protein [43,44] or stimulatory cytokines [34,45] was employed to enhance the AFP-specific responses. However, such efforts only resulted in marginal improvement of immune activation. The prime-boost immunization such as DNA prime followed by vaccinia vector [46] or adenoviral vector [47] boost generated a higher immune response to 100–200 spots/ 1×10^6 splenocytes. However, the overall antitumor effect was modest and inconsistent. In general, the HCC vaccines based on wild-type AFP demonstrated promising yet insufficient immune activation and limited antitumor responses in animal models. This weak immune activation may also explain why no H-2b restricted AFP epitopes have been identified, which could hinder the progress of HCC vaccine development.

Human studies

Butterfield and her group pioneered the AFP-based human HCC vaccine studies [48]. They first identified four HLA-A2-restricted AFP epitopes in HLA-A2 transgenic mice and in cultured human

CD8 T cells [49,50]. Butterfield *et al.* then conducted the first clinical trial in heavily pretreated stage IV patients with three biweekly vaccinations of four different AFP peptides emulsified in incomplete Freund's adjuvant [36]. They found that all six patients generated T-cell responses to most or all of the four peptides as measured by ELISPOT and tetramer assays. Thus, the human T-cell repertoire is capable of recognizing AFP in the context of MHC class I even in an environment of high circulating serum levels of AFP. In a follow-up Phase I/II trial, DCs pulsed with the AFP peptides were further used [32]. Although AFP-specific responses were detected, no significant antitumor effect was observed. It appeared that the immune responses were not potent enough or irrelevant to generate an antitumor effect. To improve the antitumor effect, an additional clinical trial was initiated to include AFP DNA vaccines followed by an adenoviral vector boost (Clinicaltrials.gov identifier: NCT00669136). In addition, cytokine granulocyte–macrophage colony-stimulating factor was also incorporated into a second new clinical trial (Clinicaltrials.gov identifier: NCT00093548). The hope is that these added measures will generate stronger immune responses and improve antitumor effects.

GPC3-based vaccines

In the late 1990s, another oncofetal protein, GPC3, was found to be highly associated with HCC [51]. In contrast to the secreted AFP, the C-terminus of GPC3 is attached to the cell membrane via a glycosphosphatidylinositol anchor. Thus, both the cellular and humoral responses can be generated against GPC3⁺ HCC tumor cells. Several anti-GPC3 antibodies for HCC immunotherapy are being developed and some have achieved clinical trial status. Readers are recommended to examine a recent review on this topic [15]. Regarding the cellular arm of GPC3-specific immune responses, both H-2b [52] and H-2d [53]-restricted CD8 epitopes have been identified in mice and have been employed to activate mouse GPC3-specific T-cell responses in order to generate an antitumor effect. Human HLA-A2 (GPC3_{144–152}) and HLA-A24 (GPC3_{298–306}) restricted human GPC3 epitopes have also been identified [54]. In a recent clinical trial [55], immunization with GPC3_{144–152} and GPC3_{298–306} peptides induced GPC3-specific cytotoxic T lymphocytes in 30 out of 33 advanced HCC patients; furthermore, 19 out of 33 patients had stabilized disease for longer than 2 months. Most importantly, the overall survival was found to be correlated with the frequency of cytotoxic T lymphocytes in the patients. Thus, GPC3-based HCC vaccines have shown great promise in treating human HCC.

Antigen engineering to create more effective HCC vaccines

AFP and GPC3 are self/tumor proteins being highly expressed during fetal development and gradually disappearing after birth. Thus, the high-affinity, self-reactive immune effector cells should be depleted in the thymus during development through the mechanism of central tolerance. Previous studies demonstrated that immunization with native self/tumor antigens is usually unable to activate the host T-cell repertoire [56–58]. Consistent with these reports, immunization with vaccinia vector-expressing, native, full-length mouse AFP was incapable of inducing AFP-specific CD8 responses in mice [59]. We reason that in order to effectively activate immune effector cells that recognize and kill tumor cells, engineering of the weakly immunogenic self/tumor proteins to create highly immunogenic antigens may be required.

Altered peptide ligands induce more robust immune responses

The ‘altered peptide ligand (APL) (or heteroclitic peptides)’ approach has been extensively studied [58,60–62] as a means of activating self/tumor antigen reactive T cells. The principle of APL design is to increase the stability of the APL and MHC complexes [63], which can then optimally engage with TCR to activate cognate T cells. In the design of APLs, the changes in the peptides must be significant enough to activate the T-cell clones that cannot be activated by a native antigen, but at the same time, the change must be subtle enough to allow the activated T cells to cross-recognize the native self/tumor antigen. However, even with careful design, the T-cell repertoire activated by APLs may be skewed [64] and the quality of clonotypic T cells may be suboptimal [65] in generating an antitumor effect. Furthermore, some of the synthetic short peptide epitopes predicted by computer may not indeed be processed and presented by target tumor cells [50]. These drawbacks of APLs may explain why recent APL clinical trials failed to show significant antitumor effects [66]. To our best knowledge, there is only one report demonstrating that the APL technology was used to activate AFP-specific T cells [67]. Unfortunately, no antitumor effect was reported in the study.

Gene vaccine encoding epitope-optimized antigen (heteroclitic gene vaccine) activates potent CD8 responses to generate strong antitumor effect

To enhance the tumor antigen-specific CD8 responses that are correlated to the antitumor effect, we reasoned that gene vaccines encoding epitope-optimized tumor antigens (heteroclitic gene vaccine) may produce a better chance of success than APLs (Figure 2). Unlike

exogenously APL short peptides, gene vaccines in the format of recombinant virus may better recapitulate natural antigen processing and presentation. The tumor antigen expressed from gene vaccine and synthesized within antigen-presenting cells will probably traverse the same processing and presentation pathways as the native antigen expressed in target tumor cells. Thus, any immune cell activated by gene vaccine will probably recognize target tumor cells. In addition, the full-length antigen offers a much broader immune response than shorter APLs. It may have a better chance of generating an antitumor effect. Furthermore, one single immunization will allow us to examine immune responses against multiple epitopes, facilitating identification of antigen-specific immune responses. Consistent with this reasoning, it was found that plasmid DNA or recombinant lentivector encoding the full-length melanoma antigen of tyrosinase related protein 1 (TRP1), which contained multiple optimized CD8 epitopes, stimulated potent TRP1-specific CD8 responses [56,57]. One single immunization with recombinant lentivector encoding the optimized TRP1 full-length antigen induced approximately 10% of circulating CD8 T cells to secrete IFN- γ in responses to native TRP1 antigen [57]. Thus, epitope-optimization of self/tumor antigen together with genetic immunization presents an effective approach to induce potent immune responses against self/tumor antigens.

Engineering AFP to create highly immunogenic HCC vaccines

Recently, using the strategy of epitope optimization and recombinant lentivector-mediated genetic immunization, we systematically studied whether HCC-associated self/tumor antigens of AFP can be engineered to become highly immunogenic so as to activate potent CD8 immune responses [68]. One major finding of our study is that the creation of epitope-optimized AFP is absolutely required to effectively activate AFP-specific CD8 T cells in mice. Once activated, however, these CD8 T cells induced by epitope-optimized AFP could efficiently cross-recognize native AFP epitopes on HCC tumor cells to generate a potent antitumor effect. In murine HCC models, including the carcinogen-induced autochthonous HCC model, we reported several important findings.

Epitope optimization creates highly immunogenic AFP that can effectively activate AFP-specific CD8 T cells, enabling one to identify three H-2b-restricted CD8 epitopes

Using an algorithmic program developed by Guevara-Patiño and colleagues [69], we identified ten putative H-2b (5 D^b and 5 K^b)-restricted CD8 epitopes in the

Figure 2. Tumor antigen synthesis, processing and presentation by an antigen-presenting cell after viral transduction and T-cell activation. Viral vector expressing offers several advantages in generating antitumor immunity: (1) the contact of virus optimally activates APCs; (2) multiple altered peptide ligands are integrated into the epitope-optimized heteroclitic full length Ag, activating a broader spectrum of immune responses and facilitating epitope identification; (3) Ag synthesized inside APCs may undergo the same Ag processing and presentation pathway as that in tumor cells; and (4) the activated T cells will have better chance of recognizing tumor cells. Ag: Antigen; APC: Antigen-presenting cell; ER: Endoplasmic reticulum.

AFP protein, which were then optimized to increase their MHC I binding. Using a MHC stabilization assay, we found that the optimized epitopes of mouse AFP_{212opt} and AFP_{499opt} peptides significantly increased the affinity of binding to H-2b molecules in comparison to their native counterparts. To test if the computer-designed optimized AFP could induce more potent CD8 responses than native AFP, we made a lentivector to express the full-length AFP containing all ten optimized epitopes. We chose lentivector-mediated immunization because it has been proved to be one of the most effective approaches to activate CD8 and CD4 T cells [70–75]. We found that a significant proportion of CD8 T cells from the optimized AFP immunized mice were able to produce IFN- γ after stimulation with three native AFP peptides: AFP₂₁₂, AFP₄₁₉ and AFP₄₉₉. As high as 1.66% of spleen CD8 T cells produced IFN- γ following AFP₂₁₂ peptide stimulation. By contrast, there were no IFN- γ -producing CD8 cells detected in the mice immunized with recombinant lentivector-expressing native AFP. These data indicate that epitope optimization of AFP is required to effectively activate AFP-specific CD8 T cells, which are capable of cross-recognizing native AFP epitopes. We also noticed that the three native AFP epitopes identified in the study had an intermediate affinity for H-2b molecules; this agrees with the theory that T cells

against the highest and lowest affinity antigens are deleted through positive and negative selection during thymus development [76].

CD8 T cells activated by optimized AFP recognize & effectively kill AFP⁺ tumor cells

One of the major drawbacks of APL technology is that CD8 T cells activated by APLs may not recognize antigen-expressing tumor cells because the native epitopes corresponding to the APL may not be naturally processed and presented by target tumor cells. This prompted us to study whether CD8 T cells activated by full-length optimized AFP expressed from gene vaccines could recognize native AFP epitopes on AFP-expressing tumor cells. Because the available B6 mouse HCC cell line, Hepa1-6, is MHC I-negative even after IFN- γ treatment, we utilized EL4-AFP tumor cells as surrogate target cells, similar to previously reported studies [35]. We found that the CD8 T cells activated by the optimized AFP gene vaccine could effectively recognize AFP⁺ EL4 tumor cells but not the parental EL4 tumor cells. Importantly, the CD8 T cells activated by the optimized AFP gene vaccine could effectively kill EL4-AFP target tumor cells. Even at the low effector/target ratio of 10:1, approximately 70% of EL4-AFP target cells were killed and eliminated within 6 h. These data strongly suggest that CD8

T cells activated by optimized AFP gene vaccine can recognize and kill tumor cells expressing native AFP. The data also support the hypothesis that the AFP CD8 epitopes identified in this study are presented by AFP⁺ tumor cells, making them recognizable by the activated CD8 T cells.

Immunization with optimized AFP but not native AFP gene vaccine completely protects mice from AFP⁺ tumor cell challenge in a CD8-dependent manner

We then studied whether immunization with optimized AFP gene vaccine could prevent mice from the challenge of EL4-AFP tumor cells. Compared to naive control mice, native AFP immunization did not protect mice from tumor challenge, although tumor growth was slowed, a limited antitumor effect was found similar to a previous report [35]. By contrast, one single immunization of optimized AFP gene completely protected mice from a lethal dose of EL4-AFP tumor challenge. In addition, the antitumor effect of optimized AFP gene immunization was mediated by the activated CD8 T cells as CD8 depletion completely abolished the antitumor effect of optimized AFP gene vaccine, which is consistent with previous findings [35].

Immunization with optimized AFP gene vaccine significantly reduces carcinogen-induced autochthonous HCC & increases liver infiltration of functional AFP-specific CD8 T cells

We then studied the antitumor effect of optimized AFP gene vaccine in the clinically relevant carcinogen (diethylnitrosamine; DEN)-induced autochthonous HCC model in mice. Our data showed that immunization with optimized AFP but not native AFP gene vaccine significantly reduced the number of DEN-induced autochthonous HCC tumor nodules. Furthermore, immunological analysis demonstrated that the optimized AFP gene vaccine significantly increased the liver infiltration of functional AFP-specific CD8 T cells that produced high levels of IFN- γ in response to native AFP peptide stimulation.

Prime boost with optimized AFP gene vaccine increases the AFP-specific CD8 response & antitumor effect in the autochthonous HCC model, & the liver infiltration of AFP-specific CD8 T cells

Previously, we found that prime-boost immunization markedly increased the CD8 memory immune responses and better prevented mice from developing autochthonous melanoma [74]. In this study, we also utilized a prime-boost strategy to increase the magnitude of AFP-specific CD8 responses. We found

that prime-boost with optimized AFP gene vaccine significantly enhanced the systemic AFP-specific CD8 effector responses. The mean fluorescent intensity of IFN- γ in the prime-boosted group was significantly higher compared with that of the prime-alone group, suggesting that the amount of IFN- γ produced is significantly greater. The AFP-specific CD8 responses returned to baseline 2 months after the boost. Importantly, the AFP-specific CD8 T-cell responses were markedly increased again at the age of 6 months, corresponding to the emerging DEN-induced HCC tumor and AFP expression. The spike of AFP-specific CD8 responses suggests that the AFP-specific memory CD8 T cells induced by vaccines may detect the emerging AFP antigen in the liver and may respond by expansion, which would then control autochthonous HCC growth. The mouse liver from the prime-boosted mice was infiltrated with a high percentage of AFP-specific CD8 T cells. Approximately 4.5% of the liver-infiltrated CD8 T cells were AFP₂₁₂-specific CD8 T cells by AFP₂₁₂-D^b tetramer staining. In addition, compared with the spleen, the percentage of AFP₂₁₂-specific CD8 T cells was significantly higher in the tumor, suggesting that either AFP-specific CD8 T cells are preferentially recruited into the liver tissue, or may simply reflect the fact that the AFP-specific CD8 memory T cells respond to the emerging HCC tumor cells in the liver by expansion; thus, they may be retained in liver tissue owing to the constant emerging AFP antigens. On the other hand, no AFP-specific CD8 T cells were found in the livers of native AFP gene vaccine prime-boosted mice. Thus, the creation of highly immunogenic AFP by epitope optimization is absolutely required to activate and increase liver infiltration of functional AFP-specific CD8 T cells to prevent autochthonous HCC in the DEN-treated mice.

Conclusion

In summary, three immunological approaches have been explored as adjuvant therapies to treat HCC following surgical or nonsurgical treatment in patients. These approaches include adoptive transfer of antigen-nonspecific CIK cells, antigen-undefined vaccines derived from HCC tumor cell lysate and antigen-defined cancer vaccines based on AFP and GPC3. The HCC cancer vaccines are based on native self-proteins associated with HCC (either as defined antigens of AFP and GPC3 or as undefined antigens of the total HCC tumor lysate). Owing to the self-nature of the HCC antigens, the immune activation and antitumor effects of these vaccines are weak. In order to improve the immune responses against HCC-associated antigens, we recently utilized the antigen engineering approach of epitope optimization to increase the immunogenicity of AFP and used the recombinant

lentivector to deliver the engineered vaccines. We found that epitope-optimized AFP but not the native AFP gene vaccine potently activated AFP-specific CD8⁺ T cells, which, critically, could cross-recognize native AFP peptides and kill AFP⁺ tumor cells; this generated a potent antitumor effect. More importantly, immunization with optimized AFP, but not native AFP, increased liver infiltration of AFP-specific CD8⁺ T cells and prevented the carcinogen-induced autochthonous HCC. In addition, prime-boost immunization with epitope-optimized AFP but not native AFP gene vaccine generates long-lasting CD8 memory cells that could detect and respond to evolving HCC tumor cells, enhancing the prevention of autochthonous HCC. Our animal study provides a blueprint to create effective human HCC vaccines that could produce an improved success rate than the current HCC vaccines based on native tumor antigens; this could provide a means to prevent HCC relapse after liver resection and *de novo* tumor development in high-risk populations.

Future perspective

We predict that the following aspects may become the future frontlines of HCC immunotherapy, which could generate better antitumor effects.

Develop highly immunogenic human HCC vaccines

The novel findings of epitope optimization to create highly immunogenic HCC-associated AFP antigen made in our animal study provides strong evidence that it is possible to induce more potent immune responses against self/tumor antigen and generate much stronger antitumor effect. We expect that it will be translated into human studies to develop novel antigen-specific human HCC vaccines. Both AFP and GPC3 can be targeted to increase the chance of success. Targeting GPC3 may offer additional benefits because humoral immunity may also contribute to the antitumor effect.

Combination therapy with checkpoint blockade

In the last few years, checkpoint blockade of targeting cytotoxic T-lymphocyte antigen 4 and programmed death 1 generate impressing antitumor effects [77]. Our own previous study found that programmed death 1 blockade could increase efficacy of lentivector immunization and rescue the effector function of tumor-infiltrating T cells [78]. We envision that such a nonspecific approach will generate a synergistic effect when combined with antigen-specific novel HCC cancer vaccines, not only by increasing the magnitude of immune responses, but also by removing the immune blockade at the effector phase in the tumor lesions.

Immune prevention in high-risk population

Vaccines are most effective when they are used to prevent diseases. However, it has been a challenge to employ cancer vaccines to prevent cancer development owing to the difficulty of gauging the risk/benefit ratio of cancer vaccination. HCC may offer an excellent model for testing the idea of immune prevention by cancer vaccines. HBV and HCV patients have an approximately 146-fold higher chance of developing HCC [79]. Even worse, approximately 24% of male HBV patients infected by vertical transmission develop HCC by age 70 years [80]. These patients have a >10,000-times higher risk than HBV-negative people. There is no intervention to stop the progression from chronic HBV infection to HCC. These high-risk patients, living in the fear of HCC, will probably accept HCC vaccines and should benefit from them. In addition, the lack of treatment options and high recurrence rate of HCC after liver resection will expedite translation of research bench findings to become a form of adjuvant therapy to prevent HCC relapse.

Generation of reprogrammed autologous T cells that can specifically target tumor cells

Recent studies demonstrated that the delivery of antigen-specific TCR gene into autologous T cells to create TCR reprogrammed T cells that are specific for particular tumor antigen is a potent approach to achieve an antitumor effect [81]. In addition, chimeric antigen receptor that combines the specificity of an antibody and the potent tumor killing effect of T cells is used to engineer T cells to render novel antigen specificity and tumor-killing activity to autologous T cells for adoptive cell transfer [82,83]. Such approaches have been used successfully to treat blood cancer. However, to obtain a good high-affinity TCR or antibody genes for HCC tumor antigens of AFP and GPC3, appropriate potent vaccines may be required. Thus, our innovative vaccine design is critical not only for active immunotherapy of HCC, but also for obtaining high-affinity TCR and/or antibodies for the purpose of adoptive immunotherapy.

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Executive summary

Current immunotherapy approaches for hepatocellular carcinoma

- Adoptive transfer of antigen target-undefined cytokine-induced killer (CIK) cells has been studied in clinical trials after liver resection of hepatocellular carcinoma (HCC) tumors and in combination with transcatheter arterial chemoembolization and radiofrequency ablation. Delay of HCC recurrence and diseases progression were observed in clinical trials.
- HCC vaccines: HCC cancer vaccines based on defined antigens such as α -fetoprotein (AFP) and glypican (GPC)-3 or undefined antigens of autologous or allogenic HCC tumor lysates have been used in clinical trials. However, enhancement of the immune activation is required to generate a significant antitumor effect.
 - Antigen undefined: dendritic cells pulsed with either autologous or allogenic tumor lysate were used as vaccines.
 - Antigen defined: AFP- and GPC3-based HCC vaccines have been employed.

Engineering self/tumor proteins to create highly effective HCC vaccines

- Epitope optimization was reported as an effective approach to create highly immunogenic AFP antigens in mice.
- Genetic immunization with recombinant lentivector expressing epitope-optimized mouse AFP effectively activated AFP-specific CD8 T cells that not only cross-recognize native AFP epitopes, but also recognize and kill AFP⁺ tumor cells.
- The AFP CD8 epitopes identified in this study are naturally processed and presented by tumor cells, making them recognizable to CD8 T cells activated by optimized AFP.
- The activated AFP-specific CD8 T cells play an important role in mediating antitumor effect of AFP vaccines.
- Prime-boost immunization strategy induces long-lasting memory CD8 T cells that can detect and respond to evolving autochthonous HCC tumor induced by carcinogen and thus generate potent antitumor effects to prevent autochthonous HCC in mouse models.

Conclusion

- Adoptive transfer of CIK immune cells activated *in vitro* delays HCC recurrence after surgery and enhances the outcome of conventional HCC treatments. Further understanding the mechanisms of how CIK cells work is required to improve its antitumor efficacy.
- The efficacy of current HCC vaccines based on HCC tumor lysate and native AFP (or GPC3) protein needs improvement in order to generate meaningful antitumor effects.
- Antigen engineering by epitope optimization and genetic immunization has great potential to create highly effective and antigen-defined HCC vaccines to potentially activate immune cells to enhance antitumor effects.

Future perspective

- Develop AFP- and GPC3-based human HCC vaccines by the antigen-engineering approach of epitope optimization and genetic immunization.
- Identify high-affinity TCR genes and antibody genes that may be used to create reprogrammed T cells to treat HCC patients.
- Explore combinatorial immunotherapy approach by incorporating the checkpoint blockade antibodies into HCC vaccination.
- Conduct clinical trials to study the prevention of HCC in high-risk populations by means of administering HCC vaccines.

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Papers of special note have been highlighted as:

• of interest; •• of considerable interest

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