Promising new strategies for hepatocellular carcinoma

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Hepatocellular carcinoma (HCC) is one of the most common causes of cancer death worldwide. It usually arises based on a background of chronic liver diseases, defined as the hypercarcinogenic state. The current treatment options for HCC ranging from locoregional treatments to chemotherapies, including sorafenib, effectively regulate the limited sizes and numbers of the nodules. However, these treatments remain unsatisfactory because they have insufficient antitumor effects on the large and numerous nodules associated with HCC and because of a high recurrence rate in the surrounding inflamed liver. To develop novel and promising therapies with higher antitumor effects, recent progress in identifying molecular targets and developing immunological procedures for HCC are reviewed. The molecular targets discussed include the intracellular signaling pathways of protein kinase B/mammalian target of rapamycin and RAS/RAF/mitogen-activated protein kinase, Wnt/β-catenin and glutamine synthetase, insulin-like growth factor, signal transducer and activator of transcription 3, nuclear factor-κB and telomerase reverse transcriptase, and c-MET. Immunological studies have focused mainly on target identification, T cells, natural killer cells, dendritic cells, natural killer T cells, and vaccine development.

Key words: hepatocellular carcinoma, immunotherapy, molecular target

INTRODUCTION

Hepatocellular carcinoma (HCC) occurs primarily in individuals with chronic liver diseases related to hepatitis B (HBV) or hepatitis C (HCV) virus infections, defined as the hypercarcinogenic state.1,2 The current treatment options for HCC, ranging from locoregional treatments, surgical resection, radiofrequency ablation (RFA), and transarterial chemoembolization (TACE) to chemotherapies, effectively regulate a limited quantity of small nodules.3,4 In patients with advanced stage HCC, the molecular targeted agent, sorafenib, has been shown to significantly increase overall survival (OS) and time to tumor progression.5,6 However, the antitumor effects are insufficient for effectively controlling the large and numerous nodules of HCC and for preventing a high rate of tumor recurrence in the surrounding inflamed liver after treatment.7,8 To explore new potential strategies for inducing higher antitumor effects, recent molecular and immunological research focusing on future treatments for HCC are reviewed.

MOLECULAR TARGETS

Details of recent molecular studies of HCC treatment strategies are presented in Table 1.

AKT/mTOR and RAS/RAF/MAPK pathways

Sorafenib is a RAF inhibitor and the standard first-line, systemic drug for advanced HCC.9 It activates protein kinase B (AKT) and upregulates downstream factors in HCC cells;10,11 sorafenib-resistant HCC cells have increased expression of phosphorylated AKT (p-AKT).12 A recent paper reported potential strategies using a novel ATP-competitive, pan-AKT inhibitor as a second-line treatment after the failure of sorafenib-medicated molecular targeted therapy for advanced HCC, in which the inhibition of AKT reversed the acquired resistance to sorafenib by activating the autophagic pathway.13 Cross-talk occurs between the phosphoinositide 3-kinase (PI3K)/AKT and mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK)
Activation of the AKT/mammalian target of rapamycin (mTOR) and RAS/MAPK cascades is frequently observed and associated with aggressive tumor phenotypes and poor prognosis in human HCC.\textsuperscript{15,16} In an animal model characterized by the co-expression of activated forms of AKT and RAS, concomitant activation of the AKT/mTOR and RAS/RAF/MAPK pathways is frequently observed and associated with aggressive tumor phenotypes and poor prognosis in human HCC.\textsuperscript{15,16}
The data indicated that sorafenib enhanced the antiproliferative effect of proteasome inhibitors and that the combination of these agents could be an ideal molecular targeted therapy for HCC.

Wnt/β-catenin and glutamine synthetase

Aberrant activation of the Wnt/β-catenin pathway has been observed in at least one-third of HCCs; roughly 20% of HCCs have mutations in the β-catenin gene, and more than 50% of HCC tumors display nuclear accumulation of β-catenin.28,29 Recently, proliferation of liver cancer stem cells and HCC lines were inhibited by a Wnt/β-catenin inhibitor,30 and this correlated with a decrease in the percentage of cells in S phase. In addition, expression of two well-characterized targets of β-catenin, cyclin D1 and survivin, was reduced by the inhibitor.31 A subset of HCC was characterized not only by mutations of β-catenin, but also by overexpression of glutamine synthetase. In four human HCC lines, the enzyme L-asparaginase, a glutaminolytic drug, had a significant antiproliferative effect only in HepG2 cells expressing a mutated β-catenin. The enzyme severely depleted cellular glutamine, caused eIF2α phosphorylation, inhibited mTOR activity, and increased autophagy. The results suggested that glutamine deprivation constitutes a targeted therapy for β-catenin-mutated HCC cells addicted to the amino acid.32

Insulin-like growth factor pathway

The insulin-like growth factor (IGF) signaling pathway is an important regulatory mechanism for tumorigenesis and drug resistance in HCC and many cancers.28,33 The IGF-I receptor (IGF-IR) is an important therapeutic target in solid tumors, with currently about a dozen IGF-IR inhibitors under clinical investigation.34 Activation of the IGF receptor activation is stimulated by aflatoxin B1 (AFB1), a potent carcinogen that can induce HCC.35 The effects of AFB1 were investigated on key elements of the IGF-IR signaling pathway. Aflatoxin B1 induced phosphorylation of IGF-IR, AKT, and ERK1/2 in hepatoma cell lines, and in an immortalized human liver cell line, Chang liver. Treatment of the cells with IGF-IR inhibitor abrogated AFB1-induced phosphorylation.36 Potential synergistic effects between IGF receptor inhibition and the other molecular targeted agents, sorafenib and sunitinib, were explored in HCC cells. The cellular apoptosis induced by sunitinib, but not by sorafenib, was enhanced when IGF receptor signaling activity was inhibited by an inhibitor or by knockdown. The data indicated that combination therapy of IGF receptor inhibitors with other molecular targeted agents might improve the therapeutic efficacy for HCC.37 In another study, the inhibitory effects of two antibodies against IGF-IR were explored in tumor cells. Compared to the effects of the single antibodies, the combination of
two antibodies accelerated IGF-IR downregulation and inhibited IGF-IR activation, as well as downstream signaling, particularly AKT phosphorylation. In an HCC xenograft model, the combination reduced tumor growth to a greater degree than each single antibody. The results suggested that targeting multiple, distinct inhibitory epitopes of IGF-IR may be a more effective strategy for affecting the IGF-IR pathway in cancer.

**Signal transducer and activator of transcription 3**

Signal transducer and activator of transcription 3 (STAT3) has been implicated in signal transduction by different cytokines, growth factors, and oncogenes. Activated STAT3 plays an important role in tumorigenesis through the upregulation of genes involved in anti-apoptosis, proliferation, and angiogenesis. Recent findings showed that sorafenib inhibited tumor growth through RAF-MEK-MAPK-independent pathways, that STAT3 was a major kinase-independent target of sorafenib in HCC, and that sorafenib inhibited growth and metastasis of HCC by blocking STAT3. An antiplatelet agent, 3-(5’-hydroxy-methyl-2’-furyl)-1-benzyl indazole, induced cell cycle arrest and apoptosis by activating checkpoint kinases, and enhanced chemosensitivity in HCC. Recently, a combination of sorafenib and the antiplatelet agent inhibited SHP-1 activity and the expression of p-STAT3 (Y705) (S727), p-ERK1/2, cyclin D1, and survivin in HCC cells, suggesting that the combination can target the STAT3 signaling pathway to inhibit HCC tumor growth.

**Nuclear factor-κB and hTERT**

Nuclear factor-κB (NF-κB) has been the focus of the transcription and expression of multiple genes coding for cytokines, enzymes, and molecules involved in apoptosis, proliferation, and adhesion that are part of the inflammation–fibrosis–cancer axis of the liver. Previous reports have shown that NF-κB positively regulates human telomerase reverse transcriptase (hTERT) transcription by reinforcing hTERT promoter activity. Recently, NF-κB p65 was found to regulate hTERT at the mRNA and protein levels in stimulated HepG2 cells, and dexamethasone was shown to inhibit NF-κB-mediated hTERT expression. These findings suggested that inhibition of NF-κB is a new approach for the treatment of HCC by preventing hTERT-mediated cellular immortality.

**Hepatocyte growth factor receptor**

Hepatocyte growth factor receptor (c-Met) is overexpressed at the protein level in 25–100% of HCCs. Targeting the hepatocyte growth factor/c-MET pathway in HCCs has been reported, in which three oral small molecule c-MET tyrosine kinase inhibitors, foretinib, cabozaunitinib, and tivantinib have shown acceptable toxicity and modest clinical efficacy in phase II trials in advanced HCC. Antibodies against c-MET have been studied, including a human anti-c-Met Fab fragment and scFv fragment screening from a human naive Fab library. Researchers generated a novel conjugate of a human anti-c-Met Fab fragment (MetFab) with doxorubicin (DOX). The MetFab-DOX conjugate had an antitumor effect and reduced the side-effects of free DOX in mice. It can localize to tumor tissues, and the concentration of doxorubicin in the tumor was high after MetFab-DOX treatment. Collectively, MetFab-DOX can target c-Met expressing HCC cells effectively with decreased side effects in preclinical models of HCC.

**Adenosine triphosphate**

Adenosine triphosphate (ATP) is an abundant biochemical component of the tumor microenvironment and plays an important role in host–tumor interactions. Deletion of CD39, an ectonucleotidase that regulates extracellular nucleotide/nucleoside concentrations, resulted in higher concentration of extracellular ATP and promoted the development of liver cancer. Adenosine triphosphate is a physiological ligand for the P2Y2 nucleotide receptor (P2Y2R). In recent work, the expression of P2Y2R was upregulated in HCC cells, and P2Y2R mediated the action of ATP on the proliferation and migration of live cancer cells. As chronic hepatitis induced by HBV and HCV is a major cause of HCC, ATP can be released from inflammatory cells and promotes the development and progression of tumors. Therefore, P2Y2R may be a key player in the development and progression of chronic hepatitis-associated HCC.

**Human homolog of Drosophila headcase**

In Drosophila, headcase (HECA) is critical for adult morphogenesis. The human homolog (HECA homo) is abnormally expressed in pancreatic, colorectal, and oral squamous cell carcinoma. The silencing of HECA homo significantly increases cell division and markedly increases resistance to the chemotherapeutic cisplatin. Protein–protein interactions between HECA homo and cyclin-dependent kinase (CDK)2, CDK9, cyclin A, and cyclin K have been verified. In HCC, the levels of HECA homo protein are mostly downregulated, and the HECA homo protein can slow cell proliferation primarily by blocking the cell cycle. Hence, the HECA homo protein may act as a tumor suppressor in HCC, and might be a potential

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molecular marker for diagnostic classification and targeted therapy in HCC.

IMMUNOTHERAPEUTIC STRATEGIES

Details of recent immunological studies of treatment strategies for HCC are shown in Table 2.

Target identification

Alpha-fetoprotein (AFP) is an oncofetal protein during HCC development that may serve as a target for immunotherapy. CD4 (Th1) and CD8 (Tc1) T-cell responses to a panel of AFP-derived peptides were analyzed in patients with HCC using an intracellular cytokine assay for γ-interferon (IFN-γ). Anti-AFP Th1 responses were more likely to be present in patients who were in an early stage of disease (for both tumor stage and liver cirrhosis), whereas anti-AFP Tc1 responses were more likely to be present in patients with late-stage liver cirrhosis. Therefore, these data provided valuable information for the design of vaccination strategies against HCC. Glypican-3 (GPC3) is a tumor-associated antigen that is overexpressed in HCC and is only expressed in the placenta and embryonic liver. An HLA-A2-restricted GPC3(144–152) (FVGEFFTDV) peptide has been shown to induce GPC3-reactive cytotoxic T cells (CTLs) in HLA-A2 transgenic mice. A phase I clinical trial of an HLA-A2-restricted GPC3(144–152) peptide vaccine was carried out in 14 patients with advanced HCC. An increase in the peptide-specific CTL frequency was observed in 86% of the patients after vaccination, and several peptide-specific CTL clones were established from peripheral blood mononuclear cells (PBMCs) of the vaccinated patients. These results suggested that the GPC3(144–152) peptide vaccine can induce high avidity CTLs capable of killing HCC cells expressing GPC3. Aspartate-β-hydroxylase (ASPH) is a highly conserved transmembrane protein that is overexpressed in HCC and promotes a malignant phenotype. The epitope-specific components required for a peptide-based candidate vaccine were investigated. Both HLA class I- and class II-restricted peptides derived from ASPH induced T cell activation in HCC, indicating that ASPH protein and related peptides produce the type of cellular immune responses required to generate antitumor activity. Annexin A3 (ANXA3) is preferentially expressed in cancer stem-like cells/cancer-initiating cells (CSCs/CICs) derived from HCC cells. ANXA3-transfected dendritic cells (DCs) induced functionally active T cells, and these effector T cells can kill CD133+ HCC CSCs/CICs in vitro and in vivo. These findings suggested that ANXA3 represents a potential CSC/CIC-specific therapeutic target for improving the treatment of HCC.

Cancer/testis (CT) antigens are promising target molecules for immunotherapy. To identify potential CT antigens, a testis cDNA library was immunoscreened with sera from patients with HCC by serological analysis of recombinant cDNA expression libraries (SEREX). Two antigens, A-kinase anchoring protein 3 (AKAP3) and CT1p11, were isolated from the patients by phage plaque analysis; anti-AKAP3 antibody was detected in sera from 15 of 27 patients with HCC and 8 of 27 healthy donors, suggesting that AKAP3 may be an immunogenic tumor antigen. New York esophageal squamous cell carcinoma-1 (NY ESO 1) is one of the CT antigens. T cell response was evaluated following stimulation with DCs pulsed with recombinant NY ESO 1 protein (rESO) in patients with HCC. Recombinant ESO DCs significantly stimulated T cell proliferation. The specific lysis of T cells stimulated with rESO DCs was higher in the NY ESO 1-positive HCC cells. These data suggested that NY ESO 1 might be used as a potential target for immunotherapy in advanced HCC. HBx is an oncogenic tumor-associated antigen and is dominantly expressed in hepatitis and hepatoma tissues. A study was designed to test whether a replication-defective adenovirus vaccine expressing HBx antigen could be effectively used in the immunotherapy of HCC. The adenovirus vaccine expressing HBx antigen induced protective and therapeutic antitumor immunity in the hepatoma models in immune-competent mice. These findings supported the development of adenovirus vaccines based on HBx antigen for the treatment of HBV-associated HCC.

T cells and NK cells

Antigen-specific T cell therapy, or T cell receptor (TCR) gene therapy, is a promising immunotherapy for infectious diseases and cancers. An efficient cloning and functional evaluation system was used to determine the antigen specificity of TCR cDNAs derived from single antigen-specific human T cells within 10 days. Using the system, 210 HCC-specific TCRs were obtained, and the cytotoxic activity of CTLs carrying these TCRs was revealed against peptide-bearing cells. This system may provide a fast and powerful approach for TCR gene therapy for cancers, including HCC. In a recent study, TCR αβ chain genes of AFP-specific CTLs were cloned into a lentiviral vector and linked by 2 A peptide to form a full-length TCR coding sequence. Non-specific activated T cells were engineered by lentivirus infection. The number of IFN-γ-secreting T cells and the specific cytotoxicity toward HepG2 significantly increased in vitro and in tumor-bearing NOD/SCID mice. Hepatocellular carcinoma cells often have integrated HBV-DNA and can be targeted by HBV-specific T cells. The electroporation of mRNA
Table 2  Recent immunological studies of treatment strategies for hepatocellular carcinoma

| Topics | Subjects | Years | References |
|--------|----------|-------|------------|
| Target identification | | | |
|  > Anti-AFP peptide Th1/Tc1 responses | Human | 2010 | 62 |
|  > HLA-A2-restricted GPC3 peptide vaccine | Human (phase I) | 2011 | 63 |
|  > HLA class I/II-restricted ASPH peptides | Human | 2015 | 65 |
|  > ANXA3 in CSCs/CICs | Cell, mouse, human | 2015 | 66 |
|  > AKAP3 by SEREX | Human | 2012 | 67 |
|  > DCs pulsed with NY ESO 1 | Human | 2013 | 68 |
|  > Adenovirus vaccine expressing HBx | Cell, mouse | 2010 | 69 |
| T cell and NK cell | | | |
|  > Fast cloning of TCR cDNAs | Human | 2013 | 70 |
|  > TCR αβ chain genes of AFP | Cell, mouse | 2015 | 71 |
|  > Electroporation of anti-HBV TCR | Cell, mouse | 2013 | 72 |
|  > HBsAg-specific TCR-modified T cells | Human | 2015 | 73 |
|  > Vγ9Vδ2 T cells | Human | 2010 | 74 |
|  > NK cell antitumor function by blocking STAT3 | Cell | 2013 | 76 |
|  > Allogeneic suicide gene-modified killer cells | Cell, mouse | 2014 | 77 |
| Dendritic cell preparation | | | |
|  Tumor lysate | | | |
|  > Total RNA, cell lysates, autophagosome, allogeneic fusion | Human (phase II) | 2013 | 79 |
| | Mouse, human | 2014 | 80 |
| | Cell | 2010 | 81 |
| | Cell, mouse | 2014 | 82 |
| | Mouse | 2013 | 83 |
| | Cell | 2010 | 84 |
|  > + IL-12, + activated T cell | Cell, mouse | 2014 | 85 |
| | Human (phase II) | 2012 | 86 |
| | Human (phase II) | 2014 | 87 |
| AFP | | | |
|  > rAAV, peptide, mannose receptor, TAAs | Cell | 2015 | 88 |
| | Cell, human | 2011 | 89 |
| | Cell, human | 2015 | 90 |
| | Human (phase I) | 2012 | 91 |
|  > + IL-2, + HBsAg | Cell, mouse | 2012 | 92 |
| | Cell, mouse | 2010 | 93 |
| Other | | | |
|  > GPC3, α-Gal, tumor stem cells and RNA | Cell, mouse | 2015 | 94 |
| | Human (phase II) | 2011 | 95 |
| | Human (phase I) | 2015 | 96 |
| | Cell, in vivo | 2010 | 97 |
|  > + PMWA, + cryosurgery, + TAE | Human (phase I) | 2011 | 98 |
| | Human | 2013 | 99 |
| | Human | 2014 | 100 |
| | Human (phase II) | 2011 | 101 |
| | Mouse | 2011 | 102 |
| Natural killer T cell (CIK cell) | | | |
|  > + RFA, TACE, hyperthermia | Human | 2013 | 103 |
| | Human (phase II) | 2014 | 104 |
| | Human | 2013 | 105 |

(Continues)
encoding anti-HBV TCR was used. The TCR-electroporated T cells efficiently prevented tumor seeding and suppressed the growth of established tumors in a xenograft model of HCC, suggesting a practical approach to cell therapy for HCC.72 In a patient who had undergone liver transplantation for HBV-related HCC, the viral antigens were expressed in the metastases. Then, the autologous T cells were genetically modified to express a hepatitis B surface antigen (HBsAg)-specific TCR. Gene-modified T cells survived, expanded, mediated a reduction in HBsAg levels, and recognized tumor cells without exacerbating liver inflammation or other toxicity. These results suggested the feasibility of providing autologous TCR-redirected therapy against HCC in hepatitis B-associated malignancies.73

Vγ9Vδ2 T cells show efficient lytic activity against multiple human tumor cell lines. Vγ9Vδ2 T cell expansions were carried out by coculturing PBMCs with autologous DCs pretreated with aminobisphosphonate zoledronate. The cytocotoxicity of Vγ9Vδ2 T cells against autologous tumor cells was significantly increased by pretreatment of the tumor cells with zoledronate, suggesting that the method may be eligible for the adoptive immunotherapy of Vγ9Vδ2 T cells against HCC.74 Researchers found that blocking STAT3 in HCC cells enhanced NK cell antitumor function. In the case of STAT3-blocked HCC cells, NKG2D ligands were upregulated, transforming growth factor-β (TGF-β) and interleukin (IL)-10 expression was reduced, and type I IFN was induced, thus facilitating NK cell activation. These findings indicated that blocking STAT3 in HCC cells could initiate innate immunity in vivo.75,76 In a panel of HCC cell lines, human allogeneic suicide gene-modified killer cells showed an IL-2-dependent, and non-MHC class I-restricted cytotoxicity in vitro and in vivo, mainly mediated by NK and NK-like T cells.78

Dendritic cells

Dendritic cell preparation – tumor lysate

DENDRITIC CELLS CAN be pulsed with tumor-specific antigens that stimulate antitumor immune responses. The safety and efficacy of autologous DCs

Table 2. (Continued)

| Topics                  | Subjects            | Years       | References |
|-------------------------|---------------------|-------------|------------|
| Human (phase II)        | 2010                | 109         |
| Human (phase I)         | 2013                | 110         |
| Human (phase II)        | 2014                | 111         |
| Human (phase III)       | 2015                | 114         |
| Vaccine                 |                     |             |            |
| Peptides                |                     |             |            |
| > HSP72/AFP, gp96/AFP, AFP nanoparticles | Mouse | 2013 | 115 |
|                         | Mouse               | 2012        | 116        |
|                         | Mouse               | 2015        | 117        |
|                         | Mouse               | 2011        | 118        |
| > GPC3 cDNA and peptide | Mouse               | 2014        | 119        |
|                         | Human (phase I)     | 2012        | 120        |
| > MRP3, hTERT, TM4SF5   | Human (phase I)     | 2015        | 121        |
|                         | Human (phase I)     | 2015        | 122        |
|                         | Mouse               | 2012        | 123        |
| Other                   | > GM-CSF + TGFβ, HLA class I, Listeria vaccine | Human (phase I) | 2014 | 124 |
|                         |                     | Cell        | 2010       | 125 |
|                         |                     | Mouse       | 2015       | 126 |

AFP, α-fetoprotein; AKAP3, A-kinase anchoring protein 3; ANXA3, annexin A3; ASPH, aspartate-β-hydroxylase; CIC, cancer initiating cell; CIK, cytokine-induced killer; CSC, cancer stem-like cell; DC, dendritic cell; GM-CSF, granulocyte macrophage colony-stimulating factor; gp96, glycoprotein 96; GPC, glypican-3; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; HSP, heat shock protein; hTERT, human telomerase reverse transcriptase; IL-12, interleukin-12; MRP3, multidrug resistance-associated protein 3; NK, natural killer; NY ESO 1, New York esophageal squamous cell carcinoma-1; PEG10, paternally expressed 10; PMWA, percutaneous microwave ablation; rAAV, recombinant adeno-associated virus carrying the AFP gene; RFA, radiofrequency ablation; SEREX, serological analysis of recombinant cDNA expression libraries; STAT3, signal transducer and activator of transcription 3; TAA, tumor-associated antigen; TACE, transarterial chemoembolization; TAE, transcatheter hepatic arterial embolization; TCR, T cell receptor; TGF-β, transforming growth factor-β; TM4SF5, transmembrane 4 superfamily member 5.
pulsed ex vivo with a liver tumor cell line HepG2 lysate were evaluated in patients with advanced HCC. Improvement in OS was observed. In another study, DCs were transfected with RNA extracted from HepG2 to induce the expression of specific antigens. Injection of T lymphocytes from patients with HCC and transfected DCs into SCID mice limited the growth of HepG2 tumors. These methods may have a therapeutic application in humans to reduce the recurrence of HCC. 80. Dendritic cells pulsed with HCC total RNA were compared with cell lysates to determine their ability to activate T cells. The total RNA-pulsed DCs induced effector T lymphocytes showed higher killing ability, as well as higher frequency of IFN-γ production by CD4+ and CD8+ T cells, suggesting that using total RNA may be a better choice for DC-based HCC immunotherapy. 81 Tumor lysate pulsed DCs were also transduced with IL-12-encoding adenoviruses. Intratumoral injections of IL-12-DC induced strong antitumor effects, causing complete regression in 75% of early stage tumors and in 33% of advanced tumors in murine s.c. HCC. These results suggested that additional DC stimulation with IL-12 is a promising approach for treating HCC. 82 A novel therapeutic cancer vaccine platform based on tumor cell-derived autophagosomes was developed for cancer immunotherapy. Autophagosome-pulsed DC immunization induced antitumor immunity in a humanized HCC mouse model generated by transplantation of HepG2 into BALB/c-nu mice, resulting in significant inhibition of tumor growth through a specific T cell response. 83 As autologous tumor lysate-pulsed DCs plus activated T cell transfer (ATVAC) was found to improve both postoperative recurrence-free survival (RFS) and OS in patients with intrahepatic cholangiocarcinoma, 84 ATVAC was tested in an adjuvant setting for postoperative treatment of HCC in a non-randomized controlled trial. The median RFS and OS were 24.5 months and 41.0 months, respectively, in the group receiving surgery alone (P = 0.029). These data suggested that a postoperative DC vaccine plus activated T cell transfer would be feasible and effective treatments for preventing recurrence in patients with HCC. 85 The specific antitumor responses of DCs fused with allogeneic HCC cell line were investigated in autologous tumor cells. Cytotoxic T lymphocytes induced by DC/allogeneic BEL7402 fusion cells were able to kill autologous HCC cells by HLA-A2 restricted mechanisms. These results suggested that the fusion of allogeneic HCC cells and autologous DCs may have applications in antitumor immunotherapy through cross-priming against shared tumor antigens. 86

Dendritic cell preparation – AFP

In a recent study, recombinant adeno-associated virus carrying the AFP gene (rAAV/AFP) and cancer cell lysates were used to pulse antigen-presenting DCs in order to stimulate a cytotoxic T lymphocyte (CTL) response in HCC. The rAAV/ AFP-pulsed DCs showed superiority in terms of inducing an AFP-specific MHC class I restricted CTL response. 87 Alpha-fetoprotein peptide-pulsed DCs were found to enhance NK cell activation and decrease the frequency of regulatory T cells in vaccinated HCC patients. Furthermore, recombinant adenovirus-transduced DCs, with or without maturation, were the most successful at inducing NK cell activation and regulatory T cell depletion. These results are relevant for designing DC-based vaccines in patients with HCC. 88 The AFP protein is endocytosed and trafficked in human DC using the mannose receptor (MR/CD206) as the primary uptake pathway for both normal cord blood-derived AFP and tumor-derived AFP proteins. In cells from patients with HCC, tumor-derived AFP was more immunogenic, and CD4+ T cell responses were not mannose receptor-dependent. These data allowed the correlation of antitumor immunity pathways in HCC patients with this secreted antigen. 89 Dendritic cells cotransfected with IL-2 and AFP enhanced the cytotoxicities of CTLs and increased the production of IL-2 and IFN-γ significantly, and induced antigen-specific antitumor efficacy in vivo. 90 Dendritic cells infected with the AFP gene or the HCC-related antigen (HBsAg) gene induced the cytotoxic activity of CTLs against the HBV-expressing cell line HepG2.2.15. Inhibition of tumor growth was most significant in the SCID mice model. These results suggested that a vaccination therapy using DCs co-infected with the two tumor-associated antigen genes is an effective strategy for immunotherapy. 91 To develop cancer vaccines for HCC capable of promoting potent tumor-specific T cell responses, adenovirally encoded synthetic AFP was tested. A multiple tumor-associated antigen-pulsed DC vaccine was prepared by pulsing DCs with cytoplasmic transduction peptide-attached AFP, GPC3, and MAGE-1 recombinant fusion proteins. In patients with advanced HCC, DCs were injected s.c. near the inguinal lymph nodes, followed by topical application of Toll-like receptor-7 agonists around the injection site. The feasibility, safety, and immune activity of DCs pulsed with tumor-associated antigens were confirmed in this study. 92

Dendritic cell preparation – other

Dendritic cells were transduced with the GPC3 gene (DCs GPC3) and cocultured with autologous cytokine-induced
killer cells (CIKs). It was reported that DCs GPC3 CIKs significantly enhanced the cytotoxic activity against GPC3-expressing HepG2 cells and showed significant inhibition of tumor growth in nude mice. An immunotherapy using both α-Gal epitope-pulsed DCs and CIK cells was evaluated. The therapy significantly prolonged the survival of treated patients when compared to that of the controls (17.1 months vs. 10.1 months, \( P = 0.00121 \)). Adoptive immunotherapy with DCs and effector cells prescribed after percutaneous microwave ablation for patients with HCC was safe and ameliorated the percentage of peripheral lymphocytes. To retrospectively assess the effect of comprehensive cryosurgery (ablation of intra- and extrahepatic tumors) plus DC-CIK immunotherapy in metastatic HCC, 45 patients were divided into cryo-immunotherapy, cryotherapy, immunotherapy, and untreated groups. Cryo-immunotherapy significantly increased OS in patients with metastatic HCC. These results provided a new insight into the design of personalizing adoptive immunotherapy for HCC.

In our studies, the procedures to induce DCs that efficiently function in HCV-related HCC were evaluated. The maturation of DCs with OK-432, a streptococcus-derived anticancer immunotherapeutic agent, boosted production of cytokines and chemokines, such as IL-2, IL-12p70, IFN-γ, tumor necrosis factor-α, IL-13, and macrophage inflammatory protein-1α, and restored T cell stimulatory activity of DCs in mixed lymphocyte reaction. Furthermore, OK432-stimulated DCs were infused into tumor tissues following transcatheter hepatic arterial embolization treatment in patients with HCC. Kaplan–Meier analysis indicated prolonged RFS of patients treated in this manner when compared to that of historical controls. The results suggest that a DC-based, active immunotherapeutic strategy, in combination with locoregional treatments, exerts beneficial antitumor effects against liver cancer.

The efficacy of intratumoral immunotherapy using IL12 gene therapy and DC injection was evaluated for the treatment of HCC under conditions of immunosuppression. The combined immunotherapy exerted effective antitumor effects on the immunosuppressed host, resulting in significant suppression of tumor growth and complete suppression of lung and liver metastasis. These results suggested that intratumoral neoadjuvant immunotherapy using IL12 and DC is a potent and effective strategy for controlling the recurrence of HCC in patients after liver transplantation. To suppress the recurrence of HCC after the treatments, establishing an immunotherapy to kill HCC stem cells is potentially a novel therapeutic strategy. Irradiated tumor stem cells (TSC) were incubated with autologous DC to create DC-TSC in hepatitis B-positive patients with HCC. After one course of TACE, three weekly s.c. injections of DC-TSC suspended in granulocyte macrophage colony-stimulating factor were administered. There was no increase in hepatic transaminases, hepatitis B antigens, or viral DNA, indicating that autologous DC-TSC did not exacerbate HBV in these patients with HCC.

In another study, researchers developed an immunotherapy to target CD133+ HCC stem cells. The results showed that: (i) CD133+ HCC cell RNA-loaded DCs induced special CD8+ CILs (CD133+ Huh7-CILs) in vitro; and (ii) Huh7 cell-induced tumor growth in vivo was effectively inhibited by CD133+ Huh7-CILs, indicating that HCC stem cell RNA-loaded DC vaccine may have potential in treating HCC recurrence.

Recent evidence indicated that paternally expressed 10 (PEG10) plays an essential role in hepatocarcinogenesis and development. Dendritic cells transduced with the PEG10 recombinant adenovirus effectively induced a specific CTL response against HCC, inhibited tumor growth and prolonged the life span of tumor-bearing mice, suggesting that the transduction of DCs with PEG10 provides a promising strategy for cancer immunotherapy of HCC.

**Natural killer T cells**

A subset of natural killer T lymphocytes, CIK cells, was generated in vitro by incubation of peripheral blood lymphocytes with anti-CD3 mAb, IL-2, IL-1α, and IFN-γ. The higher antitumor activity of CIK cells was mainly due to the higher proliferation rate of CD3+CD56+ cells. The biological characteristics of autologous CIK cells from patients with HCC were compared following different procedures for the separation of PBMCs. Apheresis is more effective at enhancing the antitumor efficacy of CIK cells than Ficoll lymphocyte separation. However, significant attention should be paid to the possibility of adverse reactions in apheresis donors.

In recent studies, patients with HCC were treated with CIK cells and other standard treatments. In combination with surgery, RFA, TACE, and local radiofrequency hyperthermia, CIK cell immunotherapy improved OS, PFS, or disease control rates. In addition, the application of CIK treatment was assessed using a nomogram of the benefit of adjuvant effects in patients with HCC, in which independent factors for OS were tumor size, capsule, pathological grades, total bilirubin, albumin, prothrombin time, AFP, and tumor number. More recently, a multicenter, randomized, open-label, phase III trial was carried out for the efficacy and safety of adjuvant immunotherapy with activated CIK cells. The study included 230 patients in Korea with HCC treated by surgical resection, RFA, or...
and measurable immune responses and antitumor efficacy of GPC3-derived peptide vaccination was well-tolerated, all survival was significantly longer in patients with high GPC3-specific CTL frequencies. The results indicated that GPC3-derived peptide vaccination was well-tolerated, and measurable immune responses and antitumor efficacy were noted. Multidrug resistance-associated protein 3 (MRP3) is a carrier-type transport protein belonging to the ABC transporter family. The safety and immunogenicity of an MRP3-derived peptide (MRP3765) as a vaccine were investigated. The vaccination induced MRP3-specific immunity in 72.7% of the patients. Among 12 HCC patients, one patient showed a partial response, nine showed a stable disease, and two showed a progressive disease. The median OS time was 14.0 months. Human telomerase reverse transcriptase is a catalytic enzyme required for telomere elongation. The safety and immunogenicity of an hTERT-derived peptide (hTERT461) as a vaccine were investigated. The vaccination induced hTERT-specific immunity in 71.4% of patients, and 57.1% of patients injected with hTERT461 peptide-specific T cells avoided HCC recurrence after vaccination. The results illustrated the potential of these peptides to provide clinical benefit in patients with HCC.

The transmembrane 4 superfamily member 5 protein (TM4SF5) induces uncontrolled growth of HCC cells through the loss of contact inhibition. To improve the efficacy of peptide vaccines, a peptide hTM4SF5R2–3 was formulated without carriers using the natural phosphodiester bond CpG-DNA and a special liposome complex (lipoplex (O)). Pre-immunization with the complex had prophylactic effects against tumor formation of HCC cells implanted in a mouse tumor model, suggesting a novel prophylaxis measure as well as therapy for TM4SF5-positive HCC.

Other
A phase I trial of a novel autologous whole-cell tumor cell immunotherapy was carried out, which incorporates a dual granulocyte macrophage colony-stimulating factor expression/bifunctional small hairpin RNA interference vector. This DNA targets furin, which is a proconvertase of TGF-β1 and TGF-β2. Based on the long-term follow-up, treated patients had a survival ranging from 319 to over 1043 days. Characterization of recurrent HCC cells will facilitate the design of future therapeutic strategies for recurrent HCC. Two cell lines were established from primary and recurrent tumor tissues of the same patient with HCC. Although the HCC cell line from recurrent tissues downregulated HLA class I expression, pretreatment with cytokines (tumor necrosis factor-α and IFN-γ) increased the expression of HLA class I molecules, and rendered them more susceptible to CD8+ T cell-mediated recognition in vitro. This strategy may be an effective therapeutic approach for preventing HCC recurrence and for controlling recurrent HCC growth.

The ability of the attenuated HCC-specific Listeria vaccine was tested in a mouse model. Immunization with the vaccine caused a strong antitumor response, especially in mice reinfused with DCs. These results suggested that the

Vaccines
Peptides
By way of glutaraldehyde cross-linking with AFP protein, potential therapeutic protein vaccines, heat shock protein 72/AFP and glycoprotein 96/AFP, were constructed. The vaccines acted synergistically to significantly increase the AFP-specific CD8+ T cell responses and produced an impressive cytotoxic antitumor effect against AFP-expressing tumors. These data suggested that tumor vaccines by cross-linking tumor antigen and heat shock protein 72 and glycoprotein 96 are promising approaches to cancer therapy. Diethylnitrosamine injected into infant mice resulted in the development of multinodular HCC in which AFP is expressed. The animals received an antigen-specific immunization with a synthetic vector consisting of a low dose of AFP-encoding plasmid formulated with the amphiphilic block copolymer 704. The AFP-specific immunotherapy led to a significant (65%) reduction in tumor size. The results supported the use of an antitumor immunotherapy based on vaccination with nanoparticles consisting of low-dose antigen-encoding DNA formulated with a block copolymer. A GPC3 cDNA vaccine was constructed by using a recombinant plasmid encoding murine GPC3 cDNA for treatment of HCC in a mouse model. Specific immune responses were detected in vitro, and homogenous tumor growth and prolonged survival time were seen in vivo, indicating that the GPC3 DNA vaccine could elicit specific and effective cellular antitumor immunity against GPC3+ HCC. In a non-randomized, open-label, phase I clinical trial, the safety and efficacy of GPC3 peptide vaccination were analyzed in patients with advanced HCC. Thirty-three patients underwent GPC3 peptide vaccination. One patient showed a partial response, and 19 patients showed stable disease 2 months after the initiation of treatment. A GPC3-specific CTL response was seen in 30 patients. Overall survival was significantly longer in patients with high GPC3-specific CTL frequencies. The results indicated that GPC3-derived peptide vaccination was well-tolerated, and measurable immune responses and antitumor efficacy were noted. Multidrug resistance-associated protein 3 (MRP3) is a carrier-type transport protein belonging to the ABC transporter family. The safety and immunogenicity of an MRP3-derived peptide (MRP3765) as a vaccine were investigated. The vaccination induced MRP3-specific immunity in 72.7% of the patients. Among 12 HCC patients, one patient showed a partial response, nine showed a stable disease, and two showed a progressive disease. The median OS time was 14.0 months. Human telomerase reverse transcriptase is a catalytic enzyme required for telomere elongation. The safety and immunogenicity of an hTERT-derived peptide (hTERT461) as a vaccine were investigated. The vaccination induced hTERT-specific immunity in 71.4% of patients, and 57.1% of patients injected with hTERT461 peptide-specific T cells avoided HCC recurrence after vaccination. The results illustrated the potential of these peptides to provide clinical benefit in patients with HCC.

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HCC-specific Listeria vaccine is a feasible strategy for preventing HCC.126

CONCLUSIONS

RECENT MOLECULAR AND immunological studies of treatment strategies for HCC were reviewed. Many intracellular signaling molecules and immunomodulatory procedures have been tested for the ability to suppress tumor growth and to improve the tumor microenvironment. Most of these techniques are promising and are promoting the development of next-generation therapies. To increase their antitumor efficacy, a future direction for the development of clinically effective cancer treatments may be not only to modify the therapies, but also to combine them with each other, and probably with immune checkpoint inhibitors. Hepatocellular carcinoma is an aggressive and treatment-resistant malignancy worldwide. Further progress in translational and therapeutic research is urgently required to reduce the suffering of patients who have this cancer.

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