Peptide-Based Vaccines for Hepatocellular Carcinoma: A Review of Recent Advances

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Abstract: Primary liver cancer is the sixth most commonly diagnosed cancer and the third leading cause of cancer-related deaths worldwide. After surgery, up to 70% of patients experience relapses. The current first-line therapy for advanced cases of hepatocellular carcinoma (HCC) comprises sorafenib and lenvatinib administered as single-drug therapies. Regorafenib, cabozantinib, and ramucirumab are administered as second-line therapies. Recently, it has been reported that using the immune checkpoint inhibitors atezolizumab (anti-PDL1 antibody) and bevacizumab (anti-VEGF antibody) leads to longer overall survival of unresectable cases, when compared with the use of sorafenib. The role of cancer immunity against HCC has attracted the attention of clinicians. In this review, we describe our phase I/II clinical trials of peptide vaccines targeting GPC3 in HCC and discuss the potential of peptide vaccines targeting common cancer antigens that are highly expressed in HCC, such as WT-I, AFP, ROBO1, and FOXM1. Further, we introduce recent cancer vaccines targeting neoantigens, which have attracted attention in recent times, as well as present our preclinical studies, the results of which might aid to initiate a neoantigen vaccine clinical trial, which would be the first of its kind in Japan.

Keywords: common cancer antigen, cancer vaccine, glypican-3, neoantigen, personalized peptide vaccine

Introduction

In 2020, there were 907,100 new cases of primary liver cancer, and the disease was the cause of death in 8.3% of the patients. These numbers make primary liver cancer the sixth most commonly diagnosed cancer and the third leading cause of cancer-related deaths worldwide. The prevalence of this disease is annually increasing owing to a higher rate of diagnosis as well as the longer life expectancy of the patient. Risk factors, leading in most of cases to a cirrhosis state, are hepatitis B and C type (which are viruses widely spread in Asia and Africa), fatty liver disease including nonalcoholic fatty liver diseases and NASH (nonalcoholic steatohepatitis) and alcoholic liver diseases. Cases of food contamination by the mycotoxin B1 aflatoxin produced by Aspergillus or industrial pollution have also been reported to contribute to the development of hepatocellular carcinoma (HCC). HCC has a high recurrence rate, ranging between 40% and 70%, and it is resistant against several standard therapies; therefore, it is classified as a refractory cancer. Early diagnosis leads to a 5-year survival rate higher than 70% when surgical resection or local radiofrequency ablation is applied. However, when the cancer is at a later advanced stage or is unresectable, the 5-year survival rate is lower than 16%. Sorafenib is the first line of treatment; it is very effective at early...
stages of the disease, but it efficacy reduces as the disease progresses.\(^5\) However, as sorafenib administration can lead to drug-resistance acquisition, favoring the growth of resistant tumor clone cells, the drug lenvatinib is also frequently used as the first-line treatment.\(^6\) Even though lenvatinib is not more efficient than sorafenib in terms of overall survival, lenvatinib is associated with a higher overall response rate and progression-free survival than sorafenib. In addition, new immunotherapy strategies are emerging as robust candidates for the treatment of HCC. Administration of small molecule inhibitors of multiple receptor tyrosine kinases in combination with immune checkpoint inhibitors (ICIs) has been reported to be effective in other cancers and is currently under investigation as a treatment option for HCC.

Immunotherapy approaches have been reported to be efficacious for various cancers.\(^7\) In addition, the idea of using of our own immune system to fight the disease is positively perceived and easily understood by the public. Immunotherapy is frequently used in combination with radiotherapy or chemotherapy or as the last resort if the two therapies have failed. The main immunotherapies already used in a clinical setting comprise ICIs.\(^8\) These compounds target immune checkpoint molecules present on the surface of cells, which physiologically restrict the risk of an auto-immune response after immune activity. However, cancer cells take advantage of these checkpoints and thus remain protected against tumor-specific T cells. PD1/PD-L1\(^9\) and CTLA-4\(^10\) are the most well-known checkpoint receptors and are highly expressed in the membrane of T cells and tumor cells. In cancer patients, T cells frequently remain in an “exhausted” state,\(^11\) which implies that they have been activated and stimulated at a low level by the tumor antigens but could not induce a strong immune response due to the “safe” control by Treg cells. Hence, as tumor cells express immune checkpoints in high quantities, they are easy targets for ICI. A study showed that immunotherapy can be beneficial for HCC, as administering a combination of atezolizumab (anti-PDL-1) and bevacizumab (anti-VEGF) to unresectable HCC cases led to increased overall survival, longer median survival, and increased overall response rate in comparison with administering sorafenib.\(^12\)

To further understand immunotherapy and its efficacy, it is necessary to consider the inter-individual differences in disease responses. Each person has their own “immunity ID” composed of the Human Leukocyte Antigen (HLA) system,\(^13\) a complex expressed at the surface of most molecules of MHC I, encoded by 6 different alleles, and MHC II, encoded by eight different alleles. The MHC system is the main guard against self-aggression of our bodies. Peptides that result from degradation of self or exogenous proteins are presented by MHC and recognized by some T cells, using a surface receptor system named T-cell receptor (TCR). In the case of a matched MHC-peptide-TCR combination, T cells are activated and start an expansion phase, which takes several days. CD4 T cells recognize MHC class II and help other immune cells by enhancing their immune response secretion factors, such as IFN-\(\gamma\) and interleukins.\(^14\) CD8 T cells present cytotoxic activity,\(^15\) which destroys the cells presenting the recognized peptides. Previous studies have reported the presence of antigens specifically expressed by cancer cells, termed “cancer antigens,” which have the potential to be the targets that are recognized by T cells, and used for cancer treatment. In this review, first, we summarize the “cancer antigens” expressed in HCC and then review the future development and potential of cancer vaccines targeting these cancer antigens based on our experience in clinical and preclinical studies.

### Cancer Antigen Classification

The most commonly used cancer antigen classifications are listed in Table 1.\(^16\)\(^-\)\(^25\) To date, more than 200 cancer antigens have been identified. The antigens and their T cell epitope presented were obtained from TANTIGEN 2.0: Tumor T-cell Antigen Database (met-hilab.org) and Cancer antigen peptide database (https://caped.icp.ucl.ac.be/).

Common cancer antigens (CCAs) can usually be organized into three categories: tumor-associated antigens (TAAs), antigens derived from gene mutations in tumors, and virus-derived antigens.\(^26\) TAAs are usually overexpressed in tumors compared with those in normal tissues; therefore, they are also used as diagnostic markers. This is the case for prostate-specific antigen, carcinoembryonic antigen, and alpha-fetoprotein levels in serum. Since some TAAs are also expressed in normal cells at a lower level, targeting them with immunotherapy presents the risk of triggering an auto-immune response. Tumor-specific antigens (TSAs) originate from oncogenic drivers with non-synonymous mutations, leading to modified peptides or “neoantigens.” Therefore, TSAs are expressed exclusively in tumors and are not found in healthy tissues. TSAs serve as ideal targets for diagnosis and immunotherapy because of their tumor specificity and the absence of immune tolerance. The interest of using the mutated
antigen to increase the immune response in comparison with the wild-type antigen for diagnosis or immunotherapy has been known for a long time.\(^\text{27}\) However, neoantigens are, in general, patient-specific as a result of the tumor heterogeneity and the inter-individual mutations observed in all cancers. Developing and using therapy approaches

Table 1 The Classification of Cancer Associated Antigens and Their Expression in Cancers

| Category                                | Antigens | Antigens Characters                                                                                                                                                                                                 | HCC Expression | Ref |
|-----------------------------------------|----------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----------------|-----|
| Cancer/Testis (CT) antigens             | MAGE     | These antigens are expressed in cancer tissues and normal tissues only in testis, ovary, and placenta. Although the frequency of patients who have the expression of these antigens is not high, they also are expressed in a variety of cancers, therefore, may be applied to many cancers as a target of anti-tumor immunity. | X              | [16]|
|                                         | NY-ESO-1 |                                                                                                                                                                                                                     | X              | [17]|
| Cancer/Fetal antigens                   | CEA      | These antigens are expressed in cancer tissues and only normal fetal tissues. CEA was first discovered as cancer fetal antigen, and used as serum marker for cancer diagnosis. Not only cancer patients, these serum biomarkers are detected in other diseases, such as inflammation and diabetes. | X              | [18]|
|                                         | GPC3     |                                                                                                                                                                                                                     | X              | [35]|
| Differentiation antigens                | Tyrosinase | Differentiation antigens are specifically expressed on cancer cells and in normal tissues where cancer develops. MART-1 was well known differentiation antigen that was first identified in melanoma by Boon et al. If these antigens are also expressed in normal organs, it may cause damage to those organs. |                |     |
|                                         | gp100    |                                                                                                                                                                                                                     |                |     |
|                                         | Melan-A (MART-1) |                                                                                                                                                                                                                   |                |     |
|                                         | PSA      |                                                                                                                                                                                                                     | X              | [19]|
| Antigens associated with genetic abnormalities |         | Cancer is a disease caused by the accumulation of genetic abnormalities, and the gene products derived from these abnormalities have been identified as cancer antigens. Genetic abnormalities that occur infrequently are not useful in terms of versatility, but in recent years, these abnormalities have attracted considerable attention as cancer-specific mutant antigens (neoantigens). |                |     |
| Somatic mutation                        | TP53     | Antigens associated with somatic gene mutations in cancer                                                                                                                                                            | X              | [20]|
|                                         | EGFR     |                                                                                                                                                                                                                     | X              | [21]|
| Over expression (copy-number amplification) | HER2     | Antigens due to the amplification or overexpression of gene products                                                                                                                                               | X              | [22]|
|                                         | hTERT    |                                                                                                                                                                                                                     | X              | [23]|
| Alternative splicing                    | Survivin-2B | Antigens associated with alternative splicing                                                                                                                                                                      | X              | [24]|
| Fusion genes                            | bcr/abl  | Antigens associated with fusion genes                                                                                                                                                                              |                |     |
| Viral antigens                          | HPV E6   | Antigens derived from viral genomes. Human T-cell leukemia virus (HTLV), Epstein-Barr (EB) virus, which is associated with Burkitt's lymphoma and head and neck cancer, and human papillomavirus (HPV) are known to be associated with carcinogenesis, and to be recognized by the immune system as cancer rejection antigens. | X              | [25]|
|                                         | HPV E7   |                                                                                                                                                                                                                     | X              | [25]|
targeting neoantigens have several important requisites: 1) Neo-peptide sequences need to be predicted using sequencing technology and bioinformatics approaches; 2) The binding affinity for MHC molecules needs to be determined (increased or decreased); 3) The power of the immune response or antigenicity needs to be decreased. The disadvantages of these strategies are the high tumor mutation burden and the low number of neoantigens.

Another type of TSAs are oncogenic viral antigens. Various viruses successfully infect human cells in a persistent manner and sometimes may integrate in the host DNA, such as the human papillomavirus or the Epstein–Barr virus, or remain in the cytoplasm as RNA forms, such as the hepatitis C virus. These viruses frequently inactivate the guardians of the genome p53 and Rb, preventing apoptosis and causing the immortalization of cells. This could lead to the production of virus-induced tumor cells. These infected cells produce specific proteins and peptides that are recognized as foreign antigens, such as HBsAg (Hepatitis B), p24 (HIV), and pp65 (cytomegalovirus).

**Common Cancer Antigens in HCC**

In the following section, we describe some well-studied CCAs, such as GPC3, AFP, WT-I, FOXM1, and ROBO1, which are overexpressed in HCC, which could be used for peptide vaccines using predicted or isolated peptides. CAAs in HCC are overexpressed, meaning that in comparison with healthy tissues, the tumor cells produce a lot of mRNA and proteins of these CAA. Consequently, it is considered that around 1 peptide for 10,000 degraded proteins is presented to MHC and that the production of MHC-I peptides is correlated to the mRNA quantity, it is likely that overexpressed CAAs are good to be considered as onco-antigen candidates for cancer vaccine.

**Glypican-3 (GPC3)**

In physiological conditions, GPC3 regulates cell division and growth regulation through binding to growth factors via heparan sulfate chains. In addition, GPC3 is involved in pathological conditions, such as the Simpson-Golabi-Behmel syndrome, a genetic disease characterized by growth anomalies. Furthermore, GPC3 is highly expressed in some cancers, such as melanomas, ovarian clear cell carcinoma, lung squamous cell carcinoma, hepatoblastoma, nephroblastoma, yolk sac tumor, and hepatocellular carcinoma (HCC). Importantly, high GPC3 expression levels are associated with a poor prognosis in HCC. Because the expression of GPC3 is not observed in most normal tissues, except for the placenta, fetal liver, and fetal kidney, GPC3 is considered an ideal tumor-associated antigen to develop a cancer vaccine.

**Alpha Fetoprotein (AFP)**

AFP is highly expressed in fetal tissues, but it is rarely detected in adult tissues. AFP expression is commonly used for testicular cancer diagnosis and HCC, with levels higher than 400 μg/mL for an HCC diagnostic. As AFP is specifically overexpressed in HCC, targeting this antigen stands as a promising strategy. Recently, the interest of using high-avidity TCRs to induce an efficient anti-tumor response in HCC has been recently studied in a Japanese Phase I clinical trial. In this study, two AFP-derived peptides (AFP357 and AFP403) were injected into 15 patients with HCC, the toxicity of the treatment and the tumor evolution were monitored, and the high-avidity TCR of a patient who responded positively was cloned. Later on, the same team studied the potential of 14 predicted and AFP-derived peptides restricted to HLA class II, using the peripheral blood mononuclear cells from 47 patients with several types of liver cancer, including HCC. Four peptides showed interesting immune responses. Among them, AFP1 was the most active and presented a strong avidity for HLA-DR. Furthermore, patients expressing this peptide presented an increased survival. After HCC treatment, an increased frequency of peptide-specific T cells was observed in some patients with HLA-DRB1*1502, *0405, and *0901 alleles, demonstrating the interest of not only focus on class I but also research on the class II for a successful immunotherapy. Finally, it is important to mention that the AFP1 peptide is also presented by MHC class I, thus indicating the importance of developing immunotherapy considering the whole immune system.

**Wilms’ Tumor-1 (WT-1)**

Wilms’ tumor 1 (WT1) antigen, a part of a transcription factor encoded by a 50 kb gene that was discovered in 1990, is important for the development of the urogenital system. As a result of different mRNA splicing events, alternative start codons, and RNA editing, there are 24 isoforms of WT1, which have different functions. Similar to GPC3, WT1 is expressed in fetal tissues but not in healthy adult tissues. However, its expression is observed

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**References**

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in chronic hepatitis and cirrhotic liver, both of which are associated with HCC. WT1 overexpression is oncogenic and is associated with poor prognosis in breast cancer and leukemia. In addition, a recent report detected the overexpression of the WT-1 factor in 95% of the tumors with worst prognostic analyzed. Furthermore, WT-1 overexpression is known to promote cell dedifferentiation and resistance to chemotherapy.

Forkhead Box M1 (FOXM1)

FOXM1 is a proliferation-associated transcription factor that has four different isoforms. FOXM1 expression is a sign of active proliferation, such as that occurring in progenitor cells or regenerating tissues and also in several cancers. Overexpression of FOXM1 is associated with gene instability, cessation of senescence, and resistance to endocrine therapies in breast cancers. In addition, FOXM1 upregulates CCNB1, enhancing the proliferation of cells. FOXM1 is overexpressed in HCC when compared to that in non-tumor tissue, and its expression levels are correlated with tumor stage and tumor size. Hence, targeting FOXM1 is a suitable approach for a peptide vaccine. Some experiments have already been performed using pulsed dendritic cells with a cytoplasmic transduction peptide fusion protein of FOXM1 to evaluate the induced anti-tumor activity in HCC. In comparison with the control, a tumor regression with specific CTL activity was observed in the peptide condition. Furthermore, another study demonstrated that the use of FOXM1 predicted peptides to stimulate specific CTLs in vitro with human cells, confirming the potential for targeting this antigen.

Roundabout Homolog-1 (ROBO-1)

ROBO-1 was initially discovered in Drosophila, after which Robo homologs have been discovered in several species, including humans. Robo belongs to the immunoglobulin superfamily of cell adhesion molecules, and its ligand is SLIT. Physiologically, these receptors are important for axon guidance in the ventral midline of the neural tube during neural development. ROBO-1 has been described to have an antitumor effect in breast cancer and pancreatic ductal adenocarcinoma, but it is overexpressed in HCC. The overexpression of ROBO-1 on the cell surface in HCC makes it an interesting target for CAR-T cell therapy as well as peptide-based vaccines.

Development of Cancer Vaccine for HCC

Historically, large-scale vaccination started after Edward Jenner’s experiment in the 18th century. Owing to vaccination, poliomyelitis, rinderpest and smallpox has been almost eradicated, and the number of people infected from diphtheria, tetanus, or pertussis has greatly diminished. The discovery of pathogens and a deep understanding of the functioning of the immunological system have helped unravel the mechanisms underlying protection immunity and the development of vaccines. How to efficiently establish a long-term and potent immunological memory against pathogens has been a critical research topic for a long time, including matters such as what kind of vectors and adjuvants, as well as the optimal type of vaccines, can best induce an effective immune response. DNA vaccines, consisting of a DNA plasmid encoding a certain antigen, have gained increasing attention over time. DNA vaccines can be directly injected into a specific site of the body, where APCs or other targeted cells take up the plasmid DNA and produce and expand the antigen. Another type of DNA vaccine that has been used for vaccination is the adenovirus, which allows the antigen genes to integrate into the host genome, and hence, the replication and expression of the gene at the same time as the cells do. However, these strategies involving the integration of a gene into the host genome convey the risk of inducing a mutation in the host genome. Hence, vaccines based on the transient expression of genes, inactivated pathogens, RNA, antigen proteins, or antigen peptides have been recently developed. Materials that are used as adjuvant for vaccine were summarized in Table 2, which is adapted from the review of Bonam et al, with new additions post-2015 made with the selection list using google patents and the terms, “adjuvant”, “vaccine” and “cancer”, granted between 2015 to 2021 in US (USA) and EP (European).

In the 20th century, the identification of “cancer antigens” led to the development of vaccines to prevent, hopefully cure, cancer. Cancer vaccines need to induce a potent CTL response to regress tumor cells. In general, the antigenicity of tumor cells and their antigens is weaker than that of pathogens as a result of them originating from autologous cells. The development of cancer vaccines presents additional obstacles, including the chronic disease status of cancer patients, heterogeneity of tumor cells, and immunological tolerance against autologous tissues. A great variety of approaches have been developed to overcome these issues. Peptide vaccines are one of the...
**Table 2** Adjuvants Used for Vaccination

| Categories | Year filed/published | Composition | Patent number | Assignee |
|------------|----------------------|-------------|---------------|----------|
| **Bacteria** | 1993 | Vaccine comprises different toxin antigens | US 5182109A | National Institute of Health;The Kitasato Institute, both of Tokyo (JP) |
| toxin      | 2021 | Tetanus toxoid and CCL3 to improve DC vaccines | US-11071777-B2 | Duke University |
| bacteria body | 2005 | TB vaccine that comprises mycobacterial | PCT/EP00/01046 | Eurocine AB, Stockholm, Solna (SE) |
| 2010 | Vaccine comprises different Streptococcus pneumoniae capsular saccharide conjugates | PCT/EP08/57998 | GlaxoSmithKline Corporate Intellectual Property, North Carolina (USA) |
| 2021 | Lactic-acid-bacteria-containing composition for treating hpv infection and/or hpv-associated tumors, and mucosal immunity-inducing agent | EP-3254693-B1 | The University of Tokyo, Japan As Represented By Director General Of National Institute Of Health Sciences |
| 2020 | Immunomodulatory composition comprising bifidobacteria | US-10668117-B2 | Dupont Nutrition Biosciences Aps |
| **Cytokine** | 2013 | Supernatant collected from stimulated cultured human lymphocytes | PCT/US2004/005152 | HasumiLlc (Dba Shukokai International) New York (USA) |
| 2003 | Nerve growth factor acts as an adjuvant to enhance effectiveness of vaccine | PCT/US98/08652 | Protection Unlimited, Inc., Wilmington, DE (USA) |
| **DNA** | 2002 | Unmethylated CpG ODN and non-nucleic acid adjuvant | US 6,406,705 B1 | University of Iowa Research Foundation, Iowa City (USA); Coley Pharmaceutical GmbH, Langenfeld (DE); Ottawa Health Research Institute, Ottawa (CA) |
| CpG (TLR9 agonist) | 2004 | Vaccine comprises fusion peptide synthetically derived from cytomegalovirus with CpG ODN as an adjuvant | US 2004/0101534 A1 | Rothwell Figg Ernst & Manbeck, P.C, Washington (USA) |
| 2005 | Immune adjuvant comprising approximately 10-bp single-stranded oligonucleotide with a CpG motif, produced by treatment of bacterial DNA with endonuclease | US 6,881,561 B1 | Cheil Jedang Corporation, Seoul (KR) |
| 2010 | CpG DNA | US 7,749,979 B2 | National Pingtung University of Science and Technology, Pingtung County (TW) |
| Interferon-stimulating gene 15 | 2020 | ISG15 gene nucleic acid molecule | US-10792358-B2 | The Trustees Of The University Of Pennsylvania |
| **RNA** | 2011 | The effect of poly-iCLC is a more direct antiviral and anti-neoplastic effect mediated by interferon-inducible nuclear enzyme systems. | (US) EP1778186 | Oncwire Inc.(USA) |

(Continued)
Table 2 (Continued).

| Emulsion | oil | 1996 | W/O emulsion with different surfactant and polymer ratios | EP 0781 559 A2 | Juridical Foundation, The Chemo-Sero-Therapeutic Research Institute Kumamoto-ken (JP) |
|----------|-----|------|--------------------------------------------------------|-----------------|--------------------------------------------------------------------------------------|
|          |     | 2005 | Vaccine comprises W/O/W-type oil adjuvant             | US 2005/0158330 A1 | NOF Corporation, Tokyo (JP); Juridical Foundation, Kumamoto-shi (JP) |
|          |     | 2012 | O/W emulsion comprises metabolizable oil, tocotrienol, emulsifying agent with various antigens and methods of preparation | PCT/EP2006/069979 | GlaxoSmithKline Biologicals S.A., Rixensart (BE) |

| Aluminium | 2013 | Provides improved methods for producing the aluminium adjuvant AlPO4 | US 8,540,955 B2 | Wyeth LLC, Madison (USA) |
|          | 2013 | Preparation of freeze-dried vaccine comprising an aluminium salt adjuvant, a recombinant Clostridium botulinum neurotoxin protein, and a glass-forming agent | PCT/US2008/057355 | The Regents of the University of Colorado, A Body Corporate, Denver (USA) |
|          | 2021 | Effective amount of an autoimmune antigen and an anti-inflammatory cytokine with an aluminium-based carrier | US-10940200-B2 | East Carolina University |

| Saponin | 1998 | Comprises saponin component from the bark of Quillaja saponaria Molina as an immune adjuvant | PCT/KR96/00053 | LG Chemical Ltd., Seoul, (KR) |
|         | surfactant | 2015 | Oil-based adjuvants comprise a plant-derived surfactant, such as gum arabic, an aqueous component, and an oil | PCT/US2013/034372 | Kansas State University Research Foundation, University of Tennessee Research Foundation, Manhattan (USA) |

| Glycolipid | a-GaCer | 2010 | a-GaCer-containing triazole moiety at the amide position | PCT/KR2007/006889 | SNU R&D Foundation, Seoul (KR) |
| Glycolipid | a-GaCer | 2010 | Synthetic a-C-GaCer | US 7,771,726 B2 | New York University, New York (USA); The Research Foundation of the City University of New York, New York(USA); Aaron Diamond AIDS Research Center, New York (USA) |

| Peptidoglycan TLR2 agonist | 2014 | FMDV capsid proteins VP1 and VP3 activate TLR2 | US 8,795,678 B2 | Academia Sinica, Taipei (TW) |
| Peptidoglycan TLR2 agonist | 2016 | Lipidated cysteine-based triazoles as vaccine adjuvants covered both therapeutic and prophylactic vaccines against bacterial, viral, and protozoan infections, and cancer | US2016/020078 A1 | Council of Scientific and Industrial Research, Delhi (India) |

| Glycan | 2021 | Glycan-dependent immunotherapeutic molecules | US-10925972-B2 | The Regents Of The University Of California |

| Viral (Viral protein) viral membrane | 2009 | Membrane protein from pathogens or tumor cells with amphiphilic adjuvant | PCT/NL2004/000437 | Bestewil Holding B.V., Amsterdam (NL) |
| Viral (Viral protein) influenza | 2009 | Vaccine comprises influenza viral antigen, Q521, and sterol | PCT/EP05/05786 | SmithKlineBeecham Corporation Corporate Intellectual Property, Pennsylvania (USA) |
| Viral (Viral protein) dengue viral | 2011 | Inactivated dengue virus antigen in combination with aluminum-free adjuvant | PCT/EP10/51882 | GlaxoSmithKline Biologicals S.A., Rixensart (BE) |
| Viral (Viral protein) dengue viral | 2020 | Recombinant subunit NS3 proteins + a purified whole inactivated dengue virus immunogenic composition | US-10716843-B2 | The United States Of America As Represented By The Secretary Of The Navy, The United States Of America As Represented By The Secretary Of The Army |
| Viral (Viral protein) HBV | 2014 | HBV antigen that includes cysteine | US 8,624,004 B2 | GlaxoSmithKline Biologicals S.A., Rixensart (BE) |

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most popular cancer vaccines. They consist of a peptide or a mix of linked or free peptides, combined with adjuvants that stabilize them and increase their efficacy. The specific peptide most suitable for developing a cancer vaccine is dependent on the specific type of cancer and the immunological characteristics of the patient. Despite this, peptide vaccines are frequently used for disease treatment because of their simplicity. Recent developments focused on various ways to target liver cancer. We can mention the use of a peptide vaccine cocktail with several antigens to increase the efficacy of antitumor response. The use of dendritic cells, an important platform for T cell activation, as vaccines has also demonstrated interesting results in several studies. Here, we summarize the results of our clinical trial targeting GPC3 and introduce our recent studies on the development of cancer vaccines targeting CCAs and neoantigens.

**Phase I and II Clinical Trial of Peptide Vaccine Targeting Glypican-3**

Several clinical trials have been performed and were simply summarized in the Figure 1. In 2011, we published the results of a peptide vaccine against glypican-3, which is frequently upregulated in HCC. From February 2007 to November 2009, 33 patients from the National Cancer Center Hospital East (Kashiwa, Japan) with advanced HCC were enrolled in a phase I trial to study the safety and immunogenic response of the peptide vaccine. The use of dendritic cells, an important platform for T cell activation, as vaccines has also demonstrated interesting results in several studies.

**Table 2 (Continued).**

| Other          | Latex polymer | 2010 | Composition comprises an adjuvant such as inverse latex or polymer mixed into an antigenic medium | PCT/FR08/51807 | Air Liquide USA LLC, Intellectual Property, Houston, Texas (USA) |
|----------------|---------------|------|-------------------------------------------------------------------------------------------------|-----------------|---------------------------------------------------------------|
| long-chain hydrocarbons | 2014 | Short and long-chain hydrocarbons with Coccidio | PCT/FR2011/050069 | Société d’Exploitation de Produits pour les Industries Chimiques, Paris (FR) |
| liposome       | 2014 | Liposomal vaccine formulations comprising an adjuvant and an immunogen for immunotherapy | US 8,889,616 B2 | Oncolyne Inc., Seattle (USA) |
| Non-conventional liposomes | 2021 | Saponin-free liposome | EP-3325015-B1 | Zoetsis Services LLC |
| PRRagonist      | 2021 | Use of Diprovincs, a TLR agonist | US-11040959-B2 | The Board Of Regents Of The University Of Texas System, The Scripps Research Institute |
| CD40 ligand     | 2021 | Vaccines with CD40 ligand as an adjuvant | US-10925961-B2 | The Trustees Of The University Of Pennsylvania, Inovio Pharmaceuticals. Inc. |
| Nanoparticles   | 2019 | Synthetic nanoparticles for delivery of immunomodulatory compounds | US-10300145-B2 | Massachusetts Institute Of Technology |
| TLR7/8 agonist  | 2020 | Alkyl chain modified imidazoquinoline TLR7/8 agonist compounds and uses thereof | US-10618896-B2 | Dynavax Technologies Corporation |
| 2013 | Process for stabilizing a vaccine composition with a stabilizer | EP 2 589 392 A2 | Sanfi Pasteur, Lyon (FR) |

**Note:** Adapted from Trends Pharmacol Sci. 38(9). Bonam SR, Partidos CD, Halmuthur SKM, et al. An overview of novel adjuvants designed for improving vaccine efficacy. Trends Pharmacol Sci. 771–793, Copyright (2017), with permission from Elsevier.
the Japanese population bearing HLA-A24 and 40% of Caucasian population bearing HLA-A02. This study showed, for the first time, a correlation between the frequency of peptide-specific cytotoxic T lymphocytes and the overall survival of the patients as well as the safety of the use of the GPC3 peptide vaccine (Figure 2A).

Since radiofrequency ablation leads to an enhancement of specific T cells against HCC-associated antigens or GPC3, the same team decided to perform a single-arm Phase II study, in which 41 patients were enrolled to receive a GPC3-derived peptide vaccine as adjuvant therapy. Although the primary endpoint was not successfully reached for 1- and 2-year recurrence rates, the presence of GPC3 peptide-specific CTL responses was detected in 35 of the 41 patients. It is interesting to note that two patients who presented GPC3-positive primary tumors and experienced relapses did not present GPC3 in the recurrent tumors. Indeed, the GPC3 peptide-specific CTL successfully killed the tumor cells expressing GPC3, leaving the GPC3-negative cells to expand by selective pressure. This observation highlights the necessity of using different antigen peptides simultaneously to increase the chance of tumor regression and reduce the risk of recurrence. Another important observation was the exhausted state of the specific CTLs post-vaccination, determined by PD-1 or CTLA-4 expression, which can be overcome by ICIs. Finally, another pilot study about the use of the GPC3 peptide vaccine against advanced HCC has been performed and it demonstrated the intra-tumoral infiltration of GPC3-specific peptides CTLs, the correlation between the CTLs frequency and the overall survival, the similar TCRs repertoire by comparing tumor infiltrated cells and PBMCs and they managed the isolation of GPC3 peptide-specific CTL clones.

Based on the positive results on this vaccine in the previous phase I clinical trial, we attempted to investigate the underlying immune mechanisms. For this purpose, five patients with HLA-A02 and six patients with HLA-A24 who were vaccinated with the same peptides previously described were selected. Most of the selected patients showed progressive disease and a poor response to sorafenib. Before- and after-vaccination tumor biopsies revealed the presence of GPC3-specific infiltrated CTLs, which was correlated with a better overall

| Therapy Used                                               | Ref | Therapy Used                                               | Ref |
|------------------------------------------------------------|-----|------------------------------------------------------------|-----|
| Resection + DC vaccine/CIK                                 | [68]| Human                                                     |     |
| Resection + DC vaccine/CIK                                 | [69]| Human                                                     |     |
| Ex-vivo prepared DCs (lysate pulse, cytokine cocktail stimulation) | [70]| Human                                                     |     |
| Autologous DC pulsed with liver tumor cell line lysate     | [71]| Human                                                     |     |
| Intratumoral injection of autologous DCs                   | [72]| Human                                                     |     |
| TLR4 ligand cold-inducible RNA binding protein + ICI        | [73]| Mice                                                      |     |
| Tumor RNA-Loaded Lipid Nanoparticles                       | [74]| In vitro/ Mice                                            |     |

Table 3 Examples of Other Anti-Tumor Vaccine Methods

**Observations**
- Increased mean survival of patients with advanced (stage III) HCC
- Demonstration of the safe use of DCs
- Prevented recurrence 1 y after operation
- Improved RFS and OS
- I- and 2-year survival rates were significantly prolonged
- Disease control of 67%
- Safe and well tolerated method
- Immune response observed with a disease control rate of 28%
- Up to 17 days of the persistence of DCs in tumor
- Induction of tumor specific lymphocytes
- No clinical benefits observed
- Enhanced therapeutic efficacy of ICI
- Strong exhaustion phenotype limitation of the intratumoral lymphocytes
- DC maturation, CD8+ and CD4+ activation
- Preventive and therapeutic effects in mice

**Table 3 Examples of Other Anti-Tumor Vaccine Methods**

| Therapy Used                                               | Ref | Observations                                                                 |
|------------------------------------------------------------|-----|-----------------------------------------------------------------------------|
| Resection + DC vaccine/CIK                                 | [68]| Human Increased mean survival of patients with advanced (stage III) HCC    |
| Resection + DC vaccine/CIK                                 | [69]| Demonstration of the safe use of DCs                                        |
| Ex-vivo prepared DCs (lysate pulse, cytokine cocktail stimulation) | [70]| Human Prevented recurrence 1 y after operation                              |
| Autologous DC pulsed with liver tumor cell line lysate     | [71]| Human Improved RFS and OS                                                   |
| Intratumoral injection of autologous DCs                   | [72]| Human I- and 2-year survival rates were significantly prolonged             |
| TLR4 ligand cold-inducible RNA binding protein + ICI        | [73]| Mice Disease control of 67%                                                 |
| Tumor RNA-Loaded Lipid Nanoparticles                       | [74]| In vitro/ Mice DC maturation, CD8+ and CD4+ activation                     |

Based on the positive results on this vaccine in the previous phase I clinical trial, we attempted to investigate the underlying immune mechanisms. For this purpose, five patients with HLA-A02 and six patients with HLA-A24 who were vaccinated with the same peptides previously described were selected. Most of the selected patients showed progressive disease and a poor response to sorafenib. Before- and after-vaccination tumor biopsies revealed the presence of GPC3-specific infiltrated CTLs, which was correlated with a better overall
survival. The autopsy of a patient who received two injections showed a strong immune response with infiltrating CD8+ CTLs and tumor lysis. Although the disease continued to progress, the trial proved that an immune response was induced. A clone was successfully isolated for further analysis and experiments, including development of clinical TCR-engineered T cells (Figure 2B). Moreover, we have reported important observations in a phase I clinical trial of a GPC3 peptide vaccine against advanced pediatric solid tumors, including malignant hepatoblastoma. In this clinical trial, seven of 18 patients presented hepatoblastoma with uniform expression of GPC3. Of this cohort, two patients were not evaluated, and the rest showed complete responses (CR) and did not recur for more than six years (Figure 2C). These clinical trials stand as a proof of concept that peptide vaccines targeting GPC3 are an adequate therapy to prevent relapse and have contributed to a further understanding of the mechanisms of action of the vaccine. The number of patients who entered and received GPC3 peptide vaccine was however insufficient for the consideration of the efficacy of peptide vaccine against HCC. Finally, a proof-of-concept was successfully performed using glypican-3 and additional peptides, thus paving the way for treatment using various peptides and antigens.

### Table 4 Clinical Trial of Cancer Vaccines Targeting HCC

| Target                        | Phase | Start - End | Number of Patients | Peptide                                      | Methods/Combination | Descriptions                                                                 |
|-------------------------------|-------|-------------|--------------------|----------------------------------------------|---------------------|--------------------------------------------------------------------------------|
| Ras mutation                  | 2     | Oct-97 - May-07 | 17                | Tumor Specific Mutated Ras Peptides          | IL2 or GM-CSF       | Phase II trial to study the effectiveness of vaccine therapy plus interleukin-2 and/or sargramostim in treating adults who have metastatic solid tumors. |
| AFP                           | 1 and 2 | Jan-01 - Oct-08 | 33                | AFP - Four immunodominant HLA-A*0201-restricted peptides [hAFP137-145 (PLFQVPEPV), hAFP158-166 (FMNKFIIYEI), hAFP325-334 (GLSPNLRNFL), and hAFP542-550 (GVALQTMKQ)] | Dendritic cells     | Phase I/II trial to study the effectiveness of vaccine therapy in treating patients who have liver cancer. |
| 16 common cancer antigens     | 1 and 2 | Sep-17 - Dec-19 | 22                | 16 newly discovered and overexpressed tumor-associated peptides | Novel RNA           | New cancer vaccine called IMA970A combined with CVB102, a new adjuvant for the treatment of liver cancer (hepatocellular carcinoma). |
| AFP                           | 1 and 2 | Jul-99 - Jun-02 | 6                 | AFP - Four immunodominant HLA-A*0201-restricted peptides [hAFP137-145 (PLFQVPEPV), hAFP158-166 (FMNKFIIYEI), hAFP325-334 (GLSPNLRNFL), and hAFP542-550 (GVALQTMKQ)] | Intradermal         | Phase I/II trial to study the effectiveness of vaccine therapy in treating patients who have liver cancer. |
| DNAJB1-PRKACA                  | I     | Apr-20 – Recruting for 12 | DNAJB1-PRKACA     | Nivolumab and Ipilimumab                     | Primary objective of the trial is the safety and tolerability of administering a vaccine. |
| VEGRF1, VEGFR2                | I     | 2007 - 2013 | Recruting for 9    | VEGRF1, VEGFR2                              | The purpose of this study is to assess toxicities of angiogenic peptide vaccine therapy in treating HLA-A*2402 restricted patients with advanced hepatocellular carcinoma. |
Personalized Vaccine Targeting Common Cancer Antigens

The peptide vaccine using GPC3 was insufficient to induce a complete regression of the tumors. We are currently developing novel cocktail vaccines targeting various TAAs expressed in HCC, such as FOXM1, AFP, ROBO1, WT-1, and GPC3. As mentioned above, TAAs are frequently overexpressed in tumor tissues, but with their efficacy is debatable; as they have an autologous origin, they are not completely considered abnormal by
The immune system. In addition, the risk of autoimmunity is increased. Hence, TAA-based peptide vaccines should be carefully developed, and after having performed clinical trials with GPC3, we wanted to explore other CAAs expressed in HCC to increase the efficacy spectra to kill tumor cells, therefore we have now started to research about. The scheme for the development of vaccines targeting common cancer antigens is shown in Figure 3.
Personalized Vaccine Targeting Neoantigens

“Neoantigens” are tumor-specific antigens that appear as a result of novel mutations in tumor cells. These proteins are degraded by cells with the ubiquitin-proteasome system, resulting in the production and release of several peptides, including those that present the novel non-synonymous mutation, which are termed “neoantigens”. Tumors with high tumor mutational burden (TMB) present a higher number of neoantigens. However, HCC usually has low TMB, and hence, a deep analysis of the potential mutations and the development of personalized treatment for each individual should be performed to develop vaccines based on neoantigens, instead of solely relying on TMB. Furthermore, a deep understanding of the underlying mechanism is needed, as the neoepitope generated might not correspond to the HLA of the patient, HLA allele-specific expression loss frequently occurs in tumors, and only some neoepitopes that will successfully be presented on the MHC may trigger specific T-cells if there is no strong immunosuppression. What kind of neoantigen is the best for developing a potential peptide vaccine in HCC that is able to induce immune memory still needs to be determined.

Figure 3 Scheme of personalized vaccination strategy targeting cancer associated antigens (CAAs). Personalized vaccines can be realized using a peptide prediction system for vaccines based on an off-the-shelf database of peptides derived from various types of CAAs.
To evaluate the immunogenicity of neoepitopes derived from gene mutations in HCC, we examined whether neoepitopes could induce significant immune responses in vivo by repeatable vaccination of transgenic (Tg) mice expressing human HLA. Whole exome sequencing (WES) of resected HCC tissues in patients who have HLA-A24 or A02 and
HLA typing were performed to find somatic mutations that occurred in the tumor cells. Using our original in silico prediction algorithm, neoantigens were predicted from non-synonymous somatic mutations and frameshift mutations and subsequently synthesized. Then, peptides derived from genes conserved between mice and humans were selected and used for vaccination of HLA-A24 or A2-Tg mice using poly-ICLC as an adjuvant for a total of three times. Until now we used IFA for peptide vaccines, but it was a poor immunological adjuvant, this is why we used poly-ICLC, and in parallel, we are checking which other adjuvants would be the best (CpG, mRNA…). Splenocytes were harvested from vaccinated mice, and the response of T cells to predicted peptides was assayed by IFN-g ELISPOT assay (Figure 4A and B). Positive responses to peptide injection were observed in 43% of the A24-Tg mice, and in 41% of the A02-Tg mice. These responses were mutation-specific and did not occur against the wild-type peptide (Figure 4C). These results indicate the immunogenicity of the neoantigen epitope. Our prediction pipeline was shown to be generally accurate and capable of extracting antigenic neoantigen peptides, since many or the peptides that induced an immune response were selected by the algorithm (Figure 4D). However, some of the HLA-A02 peptides showed immune activity, including peptides with low scores predicted by the algorithm. It is necessary to improve the prediction accuracy of the algorithm by integrating new information and results from further analyses. Our results demonstrated the availability of HLA-Tg mouse models to validate prediction algorithms based on physiological immune response in vivo. Moreover, these vaccination models also are useful for the check any adverse effects and off target effect by vaccination. Now, we are preparing for publication of these results.

To date, various clinical trials of cancer vaccines targeting neoantigens have been conducted, mainly by venture companies (Table 5). A vaccine with ICIs and peptides derived from DNAJB1-PRKACA fusion kinase is currently being tested for fibrolamellar hepatocellular carcinoma in Sidney Kimmel Comprehensive Cancer Center at Johns Hopkins (NCT04248569). However, since the majority of these trials are still in Phase I, the therapeutic efficacy still remains to be determined. Some studies have reported the induction of CTL and helper T cells in patients responding to the administered neoantigen vaccines. The final goal of our study was to develop adjuvant vaccines targeting TSAs to prevent early recurrence and metastasis, resulting in a better prognosis for HCC.

**Summary**

In summary, this review discusses several examples of positive results using peptide vaccines as a treatment for cancer. The induction of CTL in humans is promising. Furthermore, the technique is simple. We also described several common cancer antigens in HCC, which have been targeted in cancer treatment with peptides. Moreover, we discussed the potential for not focusing only on one cancer antigen or one peptide. Using previously reported studies, we concluded that some peptide vaccines against HCC antigens are efficacious and lead to potential tumor regression. Hence, there is a strong interest in peptide vaccine immunotherapy using a mix of common cancer antigens containing numerous and various epitopes for both HLA class I and II. However, the achieved efficacy is still partial, and so far, no total and definitive recovery has been observed. Cancers are complex from a physical molecular diversity perspective, with several mutations and adaptive mechanisms, challenging the innovation with the immunotherapies used. Advantages of TAAs-based vaccines are the density of peptides that are presented on HLA molecules by direct binding, the cost-effectiveness, the use of a peptide or a mix of peptides relevant for an immune response decreases the risk of contamination observed for inactivated or attenuated vaccines and reduces problems linked to autoimmunity and immune evasion. However, disadvantages are the necessity to use peptides having high immunogenicity, hence the interest of a higher in silico prediction or peptidome analysis, and the HLA haplotypes diversity which remains a strong challenge to design tailor-made vaccines with peptides against multiple antigens (including neoantigens). The need for strong antigens has led to intensive research on neoantigens specific to cancers, which are thought to be more immunogenic, leading to enhanced immunological responses. The use of strong adjuvants is also required for peptide vaccines. We have tried to use peptide vaccines that prevent recurrence as adjuvant therapy. For treatment of progressive cancers, peptide vaccine alone is not enough to induce regression, so it would require a peptide vaccine combined with powerful therapy such as ICI or radiotherapy. Our clinical trial suggests that the use of peptide vaccine as adjuvant therapy after surgery is good to prolong overall survival, especially for hepatoblastoma patients. Vaccine are usually used to prevent infections, so we want to use cancer vaccine to prevent recurrence or...
Table 5 The Development of Personalized Vaccines Targeting Neoantigens

| Sponsors/Locations                      | Cancer Type       | Vaccines                                           |
|----------------------------------------|-------------------|---------------------------------------------------|
| University of Pennsylvania             | USA Melanoma      | Peptide-treated DC                                 |
| Washington University                  | USA Glioblastoma  | Peptide, poly-ICLC                                 |
| Immatics, BioNTech AG                  | Europe Glioblastoma | Peptide, poly-ICLC, GM-CSF                      |
| Agenus Inc.                            | USA Advanced tumor | Peptide, QS-21, HSP70                             |
| National Cancer Institute              | USA Solid cancer  | Polyepitope RNA                                   |
| Radbound University                    | Europe Colon cancer | Frame shift-derived peptide-loaded DC             |
| National Cancer Institute (NCI)        | USA Breast cancer | Long Peptide Vaccine, Poly ICLC                   |
| TRON, BioNTech AG                      | Europe Melanoma   | Polyepitope RNA                                   |
| Dana-Farber Cancer Institute           | USA Melanoma      | Peptide, poly-ICLC                                 |
| Washington University                  | USA Breast cancer (TN) | Polyepitope DNA                                 |
| Icahn School of Medicine at Mount Sinai, Genentech, Inc. | USA Urothelial cancer Bladder cancer | Peptide, poly-ICLC, Atezolizumab |
| TRON, BioNTech AG                      | Europe Breast cancer (TN) | Polyepitope RNA                                 |
| Icahn School of Medicine at Mount Sinai | USA Solid cancer  | Peptide, poly-ICLC                                 |
| Washington University                  | USA Breast cancer (TN) | Peptide, poly-ICLC                                 |
| Advaxis, Inc. Amgen                    | USA Colon cancer, Head and Neck cancer, NSCLC | Peptide |
| Genentech, Inc.Biontech RNA Pharmaceuticals GmbH | USA Solid cancer | Polyepitope RNA, Atezolizumab                        |
| Neon Therapeutics, Bristol-Myere Squibb| USA Lung cancer Melanoma Bladder cancer | Peptide, poly-ICLC, Nivolumab                      |
| Neon Therapeutics, Inc.                | USA NSCLC         | Peptide, poly-ICLC, Pembrolizumab                  |
| Merck Sharp and Dohme Corp.            | USA NSCLC         | Peptide, poly-ICLC, Pembrolizumab                  |
| Washington University                  | USA Breast cancer (TN) | Polyepitope DNA, electroporation                  |
| MedImmune LLC                          | USA NSCLC         | Peptide, poly-ICLC, Pembrolizumab                  |
| Icahn School of Medicine at Mount Sinai| USA Glioblastoma  | Peptide, poly-ICLC, Tumor Treating Fields          |
| NovoCure Ltd.                          | USA PDAC Colon cancer | Peptide, IFA                                      |
| M.D. Anderson Cancer Center            | USA PDAC Colon cancer | Peptide, IFA                                      |
| Dana-Farber Cancer Institute           | USA Glioblastoma  | Peptide, poly-ICLC, Radiotherapy                 |
| Washington University                  | USA PDAC          | Polyepitope DNA, electroporation                  |
| Washington University School of Medicine, Children’s Discovery Institute | USA Brain tumor | Personalized neoantigen DNA vaccine              |

(Continued)
Table 5 (Continued).

| Sponsors/Locations                                      | Cancer Type          | Vaccines                                      |
|---------------------------------------------------------|----------------------|-----------------------------------------------|
| Dana-Farber Cancer Institute, USA                       | Renal cancer         | Peptide, poly-ICLC, Ipilimumab                |
| Bristol-Myers Squibb                                    |                      |                                               |
| Washington University School of Medicine, National Institutes of Health (NIH), National Cancer Institute (NCI), USA | PDAC                  | Neoantigen Peptide Vaccine, Poly ICLC          |
| Washington University School of Medicine, MedImmune LLC, USA | Renal cancer         | Neoantigen DNA Vaccine                        |
| Dana-Farber Cancer Institute, USA                       | Lymphoma             | Peptide, poly-ICLC, Cyclophosphamide          |
| Oncovir, Inc.                                           |                      |                                               |
| Neon Therapeutics, Inc.                                 |                      |                                               |
| Washington University School of Medicine, USA           | Prostate cancer (metastasis) | Neoantigen DNA vaccine, TriGrid Delivery System |
| Bristol-Myers Squibb                                    |                      |                                               |
| Prostate Cancer Foundation                              |                      |                                               |
| BJH Foundation                                          |                      |                                               |
| Washington University, USA                              | Glioblastoma         | Peptide, poly-ICLC, Ipilimumab, Nivolumab     |
| Washington University School of Medicine, USA           | Brain tumor          | Personalized neoantigen DNA vaccine           |
| Washington University, USA                              | Brain tumor          | Peptide, poly-ICLC                            |
| Washington University School of Medicine, USA           | Brain tumor          | Personalized peptide vaccine, Poly ICLC       |
| Dana-Farber Cancer Institute, USA                       | Follicular lymphoma  | Peptide, poly-ICLC                            |
| Washington University, USA                              | Follicular lymphoma  | Peptide, poly-ICLC, Nivolumab                |

Notes: No color = used peptide for vaccination, Gray fill = synthesis of nucleotides used for vaccination (DNA or RNA). As of November 2019.

de novo tumor for patient with high risk factors (for HCC or other cancers). However, these points still need deeper investigation. The main aspect remaining to be resolved for the development of successful peptide vaccines is identifying which targets are the most effective. As previously mentioned, several antigens are overexpressed and mutated, but not all lead to a complete response. One reason why these peptide vaccines failed to induce tumor regression, despite the appearance of CTL by vaccination, may be that the CAA-derived peptides used for vaccination were not presented to HLA molecules on the tumor cell surface. HLA-ligandome analysis based on the identification of amino acid residues by mass spectrometry is an interesting, attractive and important approach to identified peptides that are presented on HLA therefore may be recognized by CTLs during tumor rejection. However, proteomics analysis has issues, only some peptides might likely be ionized during mass spectrometry analysis so it is difficult to find every peptide that may be presented on HLA. We used HLA ligandome analysis to determine neo-epitopes, but it is not enough and new techniques are required. Concerning neoantigen-based immunotherapy, challenges are the still low power prediction of algorithms to efficiently predict immunogenic peptide-derived neoantigens. Also, the poor research and prediction for CD4+ T cells needs research. However, concerning low frequency neoantigens in low mutational burden tumors, recent study demonstrated the use of radiotherapy to induce mutation, therefore neoantigens. Neoantigens belong to the intra-tumor heterogeneity, therefore, it is necessary to identify all neoantigens present in tumors to get an efficient treatment. These issues need further investigation in the future.
Data Sharing Statement
The results that support the findings of Figure 4 are not publicly available due to our preliminary data. Now, we are going to submission for any public journal.

Acknowledgments
We specially thank Takashi Yamada, Kazushi Hiranuka, Noriko Watanabe, Yuji Mishima, Norihiro Nakamura, and the members of Brightpath Bio Co. Ltd (Tokyo, Japan) for the development of our prediction pipelines for neoantigen vaccines (shown in Figure 4), omics analysis with NGS, prediction of peptides, and synthesis of peptides.

Funding
This study was funded by the collaboration fee with the corresponding author and Brightpath Bio Co. Ltd (Tokyo, Japan). Although the funding body mainly contributed to the results of Figure 4, Brightpath Bio Co. Ltd had no control over the writing, or publication in this review.

Disclosure
Tetsuya Nakatsura is currently receiving royalties from Onco Therapy Science, Inc. and fundamental research funding support from Thyas Co., Ltd, BrightPath Biotherapeutics Co. Ltd. and Takara Bio Inc. Other authors declare that they have no commercial or financial relationships that could be construed as a potential conflict of interest. No potential conflict of interest was reported by the other authors.

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