Title
A pan-cancer clinical study of personalized neoantigen vaccine monotherapy in treating patients with various types of advanced solid tumors

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Running title
Neoantigen vaccination trial for advanced tumor patients

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Conflict of Interest Statement
Some of the authors are employees at Hangzhou Neoantigen Therapeutics Co., Ltd. (Hangzhou, China) as mentioned in the affiliations. Shuqing Chen is the shareholder of Hangzhou Neoantigen Therapeutics Co., Ltd. (Hangzhou, China). Fan Mo is the shareholder of Hangzhou Al-Force Therapeutics Co., Ltd. (Hangzhou, China).
Translational Relevance

Neoantigens, a class of antigens that derive from tumor specific mutations, have been long-envisioned as optimal targets to distinguish tumor cells from normal cells while bypass central immune tolerance. The aim of neoantigen immunotherapy is to train patient’s own immune system to recognize and eliminate cancer cells through the use of neoantigens. Thus, the precise identification of neoantigens becomes the foundation for the success of this precision therapy. So far, a few clinical studies have demonstrated the anti-tumor potentials of neoantigen-based personalized vaccines in cancers such as melanoma and glioblastoma. To further evaluate the anti-cancer efficacy of neoantigen vaccine for patients with advanced solid tumors, we conducted a pan-cancer clinical study of neoantigen vaccine as monotherapy. Our preliminary results have proved the feasibility, safety and efficacy of neoantigen peptide-based vaccine. In general, our data indicated the great potentials of iNeo-Vac-P01 as a competitive candidate for further development, alone or in combination with other therapies such as checkpoint blockade or radiation frequency ablation (RFA).

Abstract

Purpose: Due to their high tumor specificity and immunogenicity, neoantigens have been considered as ultimate targets for cancer immunotherapy. Neoantigen-based vaccines have demonstrated promising efficacy for several cancer types. To further investigate the anti-tumor potentials for other types of solid tumors, we designed a peptide-based neoantigen vaccine, iNeo-Vac-P01, and conducted a single-arm, open-labeled, investigator-initiated clinical trial (NCT03662815).

Patients and Methods: Personalized neoantigen vaccines were designed and manufactured according to our bioinformatics analysis results from the whole-exome sequencing (WES) of tumor and peripheral blood cell DNAs. Patients were scheduled to be vaccinated subcutaneously (s.c.) with adjuvant on days 1, 4, 8, 15, 22 (prime phase), and days 78, 162 (boost phase). Additional immunizations were administrated every 2~3 months as per patient’s potential benefit. The safety and efficacy were assessed through adverse events, progression-free survival (PFS), overall survival (OS) and other parameters.

Results: Of the 22 patients enrolled with advanced malignancies, twenty had no or mild adverse events, while two had grade 3 or 4 acute allergic reactions only after their 6th boost vaccination. The disease control rate (DCR) was 71.4%. The median PFS was 4.6 months, whereas the median OS was not reached (12-month OS=55.1%). Around 80% of individual peptides or peptide pools elicited measurable specific immune response. Additionally, our findings revealed several potential biomarkers for the prediction of better response.

Conclusions: iNeo-Vac-P01 as monotherapy is feasible and safe for patients with advanced solid tumors. It could elicit T cell-mediated immune response targeting tumor neoantigens, and might have promising antitumor efficacy.
Introduction

In the past decade, immunotherapy has attracted intensive attention as an effective alternative cancer therapy. In particular, immune checkpoint blockade (ICB) has shown remarkable clinical responses with low toxicity and few side-effects in several cancer types, including advanced non-small cell lung cancer, melanoma, bladder cancer, gastric cancer, hepatocellular carcinoma and colorectal cancer with DNA mismatch repair deficiency (1-5). Further intensive analysis of those patients with tumor regression after ICB treatment, showed that high tumor mutation burden (TMB) could be related to better prognosis. Therefore, neoantigens, derived from tumor somatic mutations, are considered as critical and optimal targets for immunological recognition of cancer cells.

It has been widely accepted that ICBs showed better clinical response in tumors with high levels of infiltrating T cells and more antigens ("hot" tumor) rather than those lacking tumor-reactive infiltrating T cells ("cold" tumor). Neoantigen vaccines are designed to present tumor-specific neoantigens and activate cytotoxic T cells to recognize and infiltrate into tumor cells, turning "cold" tumor into "hot" tumor. Also, the process involves the training of immune system to target and kill tumor cells. As generated mostly by non-synonymous mutations in cancer cells (6-8), neoantigens are exempted from central tolerance. Therefore, neoantigen vaccines are more likely to generate robust immune responses (9,10), functioning as bona fide antigens to facilitate tumor regression (11).

Therapeutic neoantigen cancer vaccines are safe, tolerable and capable of eliciting robust T-cell responses to kill tumor cells (12,13). Plenty of related clinical trials have been launched in the past 5 years. Two studies on melanoma demonstrated that neoantigen peptide or RNA vaccines could not only benefit for the regression of advanced melanoma, but also provide long-term protection against tumor relapse and metastasis (12,13). In subsequent investigation, neoantigen vaccine along with adjuvant could induce predominantly T cell responses against predicted neoepitopes in patients with newly diagnosed glioblastoma (14,15), and increase the number of tumor-infiltrating T cells (15). Additionally, adoptive transfer of mutant-protein-specific TILs has been applied to mediate complete durable regression of metastatic breast cancer. These personalized neoantigen vaccines could elicit sustained responses of T cells and display great potentials for further development (14,15).

As mentioned above, all of these exploratory studies were done with a few patients (no more than 17) of a single cancer type, and combination therapies were applied in these studies to improve patients’ response. Despite the relatively promising clinical results, the anti-tumor potential of neoantigen vaccine has not been proven in broader cancer types. In principle, the anti-tumor potential of neoantigen vaccine was not limited to certain tumor type as long as appropriate tumor neoantigens were identified. Therefore, a pan-cancer clinical study focused on neoantigen vaccine monotherapy instead of combination therapy was conducted to demonstrate the anti-tumor efficacy of neoantigen vaccine in various types of advanced solid tumors. Preliminary results indicated iNeo-Vac-P01 monotherapy could elicit specific T cell activation and induce broad spectrum of anti-tumor effects, without limitation to tumor type. Additionally, several biomarkers potentially predictive for patient’s better response were revealed.

Materials and Methods
Patients

Eligible patients were at least 18 years old with advanced malignant tumors confirmed histologically or cytologically. Patients had disease progression after two or more lines of standard treatment, at least one measurable lesion as per investigator-assessed Response Evaluation Criteria In Solid Tumors (RECIST; version 1.1); an Eastern Cooperative Oncology Group (ECOG) performance status of 0 or 1; good functioning main organs, such as heart, liver and kidney; could provide sufficient tumor tissue and blood samples for DNA sequencing assay, or qualified genome/exome sequencing data of tumor tissues and normal tissues.

Key exclusion criteria included: having other malignant tumor, except for cured basal cell carcinoma, thyroid carcinoma, or cervical dysplasia; lack of identified neoantigen in the sequencing data; received bone marrow or stem cell transplants; allergic to any drug, polypeptide, or other potential immunotherapies.

Trial design and treatment

This was a single-arm, open-label, investigator initiated clinical study at Sir Run Run Shaw Hospital, School of Medicine, Zhejiang University in China. The final study protocol was approved by Institutional Review Board and Independent Ethics Committee, and conducted in accordance with Declaration of Helsinki and the International Conference on Harmonization Guidelines for Good Clinical Practice. All patients had signed informed consent forms before treatment. The primary endpoints of the study were safety and feasibility. And the secondary endpoints were efficacy based on PFS, OS and neoantigen-specific immune responses. The safety of the study was assessed on the basis of occurrence of adverse events (AEs). The feasibility of this trial was assessed by whether neoantigen could be identified and the vaccines could be synthesized for clinical use.

iNeo-Vac-P01 comprises 5~20 peptides at lengths of 15~35 amino acids. The peptides were grouped into 2~4 pools (based on HLA typing, affinity and allele frequency) and then injected subcutaneously (s.c.) at a quantity of 0.1 or 0.3 mg per peptide in upper arms and para-umbilical area respectively. Patients were scheduled to receive iNeo-Vac-P01 with granulocyte–macrophage colony-stimulating factor (GM-CSF) as adjuvant on days 1, 4, 8, 15 and 22 (i.e. prime phase), as well as on days 78 and 162 (i.e. boost phase) (12,15-18). The adjuvant GM-CSF was injected s.c. 30 minutes before the administration of iNeo-Vac-P01 at a quantity of 40 μg per injection nearby the injection site of iNeo-Vac-P01. Additional boost vaccines might be administered depending on ethics and patients’ potential benefit according the clinical research protocol.

Clinical assessment, monitoring and follow-up in this research were conducted, including physical examination, ECOG performance, vital sign, blood test, urinalysis to assure the safety of each immunization; imaging examination at baseline and approximately every 8 weeks post-vaccination to assess clinical efficacy; and ELISpot, TCR sequence and flow cytometry (T cell subsets and cytokines) conducted pre-treatment and every 8-12 weeks after treatment for the detection of specific immune response.

Tumors were assessed by investigators according to RECIST v1.1 criterion at baseline and approximately every 8 weeks thereafter. Patients’ conditions were monitored while receiving
neoantigen vaccine treatment and every 3 months after treatment discontinuation. The related adverse events (AEs) were recorded and graded for safety evaluation according to the National Cancer Institute Common Terminology Criteria for Adverse Events (version 4.0) throughout whole treatment period.

**Generation of personalized neoantigen vaccines**

To identify mutations derived neoantigens, some tumor tissues and blood samples were obtained directly by surgery, while others were obtained by biopsy or intravenous blood sampling. WES with coverage depths of 500x for tumor and 100x for blood cells was conducted on these samples using Hiseq 4000 NGS platforms (Illumina) (AcornMed Biotechnology Co., Ltd., Beijing, China) (The raw sequence data had been deposited in Genome Sequence Archive (GSA) under accession number HRA000171 at http://bigd.big.ac.cn/gsa-human ) (19-23) . In case of unavailability of tumor fresh sampling, formalin-fixed paraffin-embedded (FFPE) samples were used instead.

The bioinformatics analysis, which consists modules of sequencing read filtering, genome alignment, mutation calling, HLA typing, MHC affinity prediction, gene expression profiling, vaccine peptide sequence design and mutation centered prioritization based on therapeutic potency, was performed by our in-house pipeline iNeo-Suite (Supplementary Methods, Supplementary Fig. S1).

To generate personalized neoantigen vaccines, the customized clinical-grade long peptides were manufactured through chemical synthesis at GMP-like standard (bacteria-free, >95.0% purity and quantities of bacterial endotoxin less than 10 EU/mg). The water solubility of peptides was tested after synthesis, followed by the removal of insoluble peptides from iNeo-Vac-P01.

**IFN-γ Enzyme-Linked Immunospot (ELISpot) Assay**

To confirm the immunogenicity of iNeo-Vac-P01, ELISpot assays were performed for each patient at a series of time points pre- and post-vaccination. Peripheral blood mononuclear cells (PBMCs) were isolated from the peripheral blood (10-30 mL) collected from each patient, and co-incubated (2*10^5 cells per well) with peptides for 16-24 hours using Human IFN-γ pre-coated ELISpot kit according to the standard protocol. An automatic plate reader with appropriate parameters was used to count the spots in ELISpot plates (Supplementary Methods).

**T cell receptor sequence**

To observe the change of T cell population after vaccination, T cell receptor (TCR) β chain was sequenced for each patient before and after vaccination. Peripheral blood (10 mL) was collected from each patient for the extraction of RNA from PBMCs. Samples were analyzed by High-throughput sequencing of TCR using ImmuHub TCR profiling system at a deep level (ImmuQuad Biotech) (Supplementary Methods).

**Multiplexed immunofluorescence**

To examine tumor infiltrated T cells, multiplexed immunofluorescence (IF) was performed by staining 4 μm thick formalin-fixed, paraffin-embedded whole tissue sections with standard primary antibodies sequentially, and paired with a unique fluorochrome before DAPI staining. Slides were
air dried, mounted with Prolong Diamond Anti-fade mounting medium (#P36965, Thermofisher), and observed with Aperio Versa 8 tissue imaging system (Leica). Sample images were analyzed using Indica Halo software (Version 2.3.2089.52) (24,25) (Supplementary Methods).

**Cytometric analysis of T-lymphocyte and cytometric bead array (CBA) analysis of cytokines**

To quantify the activation of T cells after vaccinations, patients' peripheral T cells were extracted and labeled with several antibodies for cytometry analysis of the proportions of various types of T cells. To examine the cytokines secreted from the T cells activated by iNeo-Vac-P01, the concentrations of cytokines in patients’ peripheral blood were measured by CBA according to manufacturer's protocol (Supplementary Methods).

**Statistical analysis**

Data from the patients received at least one dose of iNeo-Vac-P01 were included in the safety and clinical effects analysis. Descriptive statistics was used to determine the characteristics of baseline, and assess vaccine's safety. Disease control rate (DCR) was defined as the proportion of complete response (CR), partial response (PR) and stable disease (SD) for best clinical response. Standard RECISTv1.1 guideline was applied for the analysis of all clinical responses, except for one data showing apparent pseudo-progression, for which iRECIST was applied instead. The survival curves were plotted with GraphPad Prism 5 (v5.01).

**Results**

**Patients and demographics**

A total of 22 cancer patients with advanced-stage of various tumor types, including non-small cell lung cancer, colorectal cancer, melanoma, pancreatic cancer, biliary tract cancer, ovarian cancer, small cell lung cancer, adrenal sebaceous adenocarcinoma, breast cancer, parotid carcinoma and gastric carcinoma, were enrolled in the trial from Feb 7th, 2018. All these patients were failed to respond or unable to tolerate the standard treatment. The patient demographics, baseline disease characteristics, and previous treatments were presented in Table 1, indicating that 5 (22.72%) patients had liver metastases, 4 (18.18%) patients had lung metastases, and 6 (27.27%) patients had both, 4 (18.18%) patients had bone metastases (Supplementary Table S1). All data in this article was collected and summarized before the date of May 31st, 2019.

**Feasibility of iNeo-Vac-P01 manufacturing for pan-cancer patients**

WES was conducted on patient's tumor fresh tissue and peripheral blood cells as normal control (Supplementary Table S2). Neoantigens were predicted and prioritized using our in-house pipeline iNeo-Suite (Supplementary Methods), which leveraged information including the allelic frequency of mutation, gene expression, the affinity between the mutated peptide and HLA complex class I and II, as well as the feasibility of peptide synthesis. Clinical-grade long peptides were synthesized at lengths of 15-35 amino acids incorporating multiple neo-epitopes of both HLA class I and II (Supplementary Table S3, S4, S5 and S6). The turn-around time of the whole process was mostly between 1.5 and 3 months, and no preference in tumor type was witnessed. Totally, 91.7% (22/24) patients received iNeo-Vac-01 immunization, while two other patients dropped out due to rapid disease progression.
Although patients enrolled were bearing various types of tumors with the purity estimated by WES data ranging from 12.4% to 83.7%, sufficient neoantigens were predicted for the successful manufacture of long peptide vaccines for each patient. To be specific, there was a median of 14 long peptides immunized, which comprised a median of 10 class I neo-epitopes and 26 class II neo-epitopes per peptide. Most patients (17 out of 22) received vaccines containing more than 10 peptides (Supplementary Table S5 and S7).

Study treatment

Each patient's peptides were pooled into 2-4 groups with maximum 5 peptides per pool. The vaccine administrated subcutaneously in two flanking sites of navel and tail-end of arms following the administration of granulocyte-macrophage colony stimulating factor (GM-CSF) as adjuvant. Peripheral cytokine and T cell subtypes monitoring, TCR sequencing, IFN-γ ELISpot assay in vitro were detected at a series of time points before and after vaccination according to the study protocol and recorded in case report form (CRF).

Patients were scheduled to receive iNeo-Vac-P01 with GM-CSF as adjuvant on days 1, 4, 8, 15 and 22 (i.e. prime phase), as well as on days 78 and 162 (i.e. boost phase). Additional boost vaccines might be administered depending on ethics and patients' potential benefit (Fig. 1A). The median duration of follow-up was 9.8 months, ranging from 0.9 to 14.5 months before the deadline May 31st, 2019. 21 patients completed prime phase vaccinations except for P014 because of the withdrawal of informed consent form. 17 out of 22 patients conducted additional boost vaccines (Fig. 1A). As shown, the median duration of treatment was 4.15 months (range, 16 days to 12.1 months). Patients P005 and P015 had stable disease (SD) ever since enrolled while P005 dropped out because of grade 3 acute allergic reactions. No progress for two patients (P001 and P011) was recorded until death. Till May 31st 2019, 20 patients had ceased iNeo-Vac-P01 while the rest of 2 patients (P015, P019) were still receiving the assigned treatment.

Safety and Tolerability

Defined by NCI-CTCAE 4.03, treatment-related AEs occurred in 54.55% of the patients (Table 2). Most witnessed AEs were in grade 1 to 2, mainly including fatigue (36.36%), chill (18.18%), and fever (13.64%). However, grade 3 to 4 treatment-related acute allergic reactions were only observed in two patients (9.09%) after their 6th round of boost vaccinations, leading to their drop-out of the study. The rest cases of AE observed throughout the treatment were reversible without particular nursing or treatment. No apparent association was found between the presence or the type of AEs and the tumor type. No treatment-related serious adverse event (SAE) and death was witnessed.

Immune response

By performing IFN-γ ELISpot assay in vitro using autologous PBMCs after vaccination, T cell activation induced by iNeo-Vac-P01 was confirmed in 19 out of 21 patients. Overall, 99 out of 125 (79.2%) individual long peptides and 43 out of 51 (84.3%) long peptide pools elicited measurable peptide-specific immune response (positive results in ELISpot assay after vaccination) in 11 patients and 10 patients respectively (Supplementary Table S8). The IFN-γ spots per 10⁵ PBMCs of the peptide or peptide pool with best response had also been shown (Fig. 1B and Supplementary Table S8). All of patients showed statistical significant increase of ELISpot spots
after vaccination (p-value < 0.05), except patient P006 and P019 showed no increase after treatment mainly owning to the noisy background at baseline (Supplementary Fig. S2). Furthermore, TCR sequencing of peripheral T cells before and after vaccination revealed that there were new high abundant clones or clones with considerably increased abundance detected post-treatment in 17 (77.3%) or 12 (54.5%) patients (Fig. 1B and Supplementary Table S9).

In order to detect the activation of both CD8+ and CD4+ T cells in tumor microenvironment during treatment, multiplexed IF on FFPE samples with antibodies of CD4, CD8 and Granzyme B (Supplementary Fig. S3) was applied to analyze the tumor regions of pre- and post-vaccination FFPE samples for three patients (P008, P013 and P015). To locate the tumor regions for the analysis, H&E (haematoxylin and eosin) staining was performed to analyze the adjacent slices of all multiplexed IF samples except the post-vaccination sample of P008 (H&E staining images obtained from Department of Pathology in Sir Run Run Shaw Hospital were used instead) (Supplementary Fig. S4). As shown in Fig. 1C, the proportion of both activated CD8+ T cells (Granzyme B positive) and activated CD4+ T cells (Granzyme B positive) were found to be increased after vaccination in 3 patients (5.5-months post-vaccination for P008, 7-months post-vaccination for P013, 8-months post-vaccinations for P015), as well as the densities of these two types of T cells. These findings suggested that iNeo-Vac-P01 had the potential to activate tumor specific CD8+ T cells and CD4+ T cells, which could subsequently infiltrate into tumor tissue and generate anti-tumor effects.

**Clinical Response**

Except for Patient P005, standard RECIST 1.1 criteria were applied to all other patients during assessment. For P005, iRECIST guideline was applied as a result of his pseudo-progression. For 21 out of 22 patients, who completed 5 prime immunizations, at least one post-treatment target lesion was evaluated. Tumor reduction was observed in the assessment of 8 out of 21 patients (38.1%), with a 16.7% maximum reduction of target lesions compared to baseline (Fig. 2A and B). The calculated DCR was 71.4% (15/21). In Fig. 2B, two patients were excluded due to new lesion appearance (P002) and the progression of non-targeted lesion occurrence (P006) at first post-treatment assessment respectively. 15 out of 21 patients (71.4%) displayed SD (8 with tumor size reduction ranging from 1.9% to 18.2%, 7 with tumor size growth ranging from 1.1% to 9.0%) with a median duration of 4.4 months (ranging from 1.7 to 14.5+ months, “+” indicates some patients maintained SD at cutoff date). The median PFS of 21 treated patients was 4.6 months (95% confidence interval (CI), 2.5 to 5.2). And the estimated percentage of patients without disease progression at 6th month was 27.3% (95% CI, 10.3% to 46.8%) (Fig. 2C). 7 of all treated patients (N=22) had died. As shown in Fig. 2D, the estimated OS at 12th month was 55.1% (95% CI, 25.9% to 76.9%), while the median OS had not been reached yet (95% CI, 9.4 to not reached). In particular, no evident differences were observed between various types of cancer, which indicated that iNeo-Vac-P01 has broad spectrum anti-tumor effects.

**Potential Biomarkers for Clinical Response**

Patients with SD lasting more than 3 months were considered to have better response than the others. Several mutant genes and copy number variants detected by WES were found to be predictive for better response (Supplementary Fig. S5). The mutations of gene MUC (mucins), ZFHX3 (Zinc Finger Homeobox 3), ABL1 (ABL Proto-Oncogene 1) seemed to be associated with
faster disease progression and poor response, while copy number variations of gene TNFRSF (tumor necrosis factor receptor superfamily), SOX3 (Sex determining region Y-box protein 3) and MAGE gene families (Melanoma-associated antigens) were relatively associated with better response (Supplementary Fig. S5) (Supplementary Results).

The changes of peripheral T cells during treatment might provide potential predictive biomarkers for clinical response, since T cells play a major role in anti-tumor response. After treatment, the proportions of effector CD8+ T cells to total T cells in patients' peripheral blood samples all increased to certain extent. Although insignificantly at most time points after vaccination, the effector CD8+ T cells of patients with good response had a relatively higher boost compared to those of patients with poor response (Supplementary Fig. S6A). Similarly, the level of interferon γ (IFN-γ) in the patients' peripheral blood samples all increased at prime phase. Notably, different from patients with poor response, patients with better response maintained a relatively high level of IFN-γ at boost phase (Supplementary Fig. S6B). On the contrary, a continuous increase of Interleukin 6 (IL-6) during treatment was observed in the peripheral blood samples of patients with poor response (Supplementary Fig. S6C). These findings implied that the clinical efficacy might be predicted by the peripheral CTLs' proliferation and activation (Supplementary Results).

Case report of an advanced hepatic biliary tract cancer patient

In the case of patient 005, a 63-year-old male, initially diagnosed with intrahepatic biliary tract cancer in 2013, was treated by surgical excision in Jun. 2013 following by postoperative chemotherapy till Apr. 2014. Tumor recurrence and metastases were confirmed by computed tomography (CT) scan and pathological tissue biopsy in Apr. 2017. Then he was treated with Apatinib for six months. He enrolled in a clinical trial of a PD-1 antibody (IBI308) for 6 cycles after failing to respond to chemotherapy again, and dropped out due to progression disease (in November 2017, on a clinical trial in The First Affiliated Hospital, Zhejiang University in China).

On Mar. 22th, 2018, he started to receive iNeo-Vac-P01. The treatment scheme is displayed in Fig. 3A, including 5 prime vaccinations and 6 subsequent boost vaccinations. Compared to standard treatment scheme with only 2 boost vaccinations, 4 more boost vaccinations were added under patient's consent, based on the facts that his symptoms were greatly relieved and the level of a tumor marker (Carbohydrate Antigen 72-4) was significantly reduced. After the last boost vaccination, a grade 3~4 acute allergic reaction occurred along with clinical manifestations: nausea, vomiting and rash. Radiographic imaging was performed at 2nd, 5th, 8th, 10th and 12th months after the first vaccination. The CT scans demonstrated an evident increase of tumor size (Maximum diameter of target lesions was 122.9 mm) at 5th month compared to baseline (Maximum diameter of target lesions was 89.1 mm), and a significant shrinkage of tumor size (Maximum diameter of target lesions was 75.4 mm) at 8th month, indicating a pseudo-progression previously occurred (Fig. 3B). His disease continually maintained stable at cutoff date (over 14.5 months). Peripheral blood mononuclear cells (PBMCs) were tested for the reactivity against the immunizing peptides. Robust de novo immune response against predicted neoantigen (mutant PIGK) was generated after 8-week vaccination, analyzed using ex vivo IFN-γ ELISpot (Fig. 3C and D). TCR sequencing of peripheral T cells revealed that the abundance of two TCR clones (CASSQDSTGAVNEQFF, CAWSVGKYGDTQYF) considerably increased after vaccination. Meanwhile, a new TCR clone (CASSFITEAKNIQYD) was detected after vaccination (Fig. 3E).
These data suggest that a subset of T cells with specificities induced by iNeo-Vac-P01 can be successfully activated and kill tumor cells.

**Case report of a pancreatic cancer patient**

In the case of patient 008, a 65-year-old female, was diagnosed with pancreatic cancer in July 2016, and later subjected to surgery. Pathology showed low differentiated adenocarcinoma in the tail of her pancreas. Total of 6 cycles of post-surgery chemotherapy was conducted based on a Gisitabine regimen. In June 2017, upper abdomen magnetic resonance imaging (MRI) enhancement scan showed relapse with hepatic metastases. Soon afterwards, she received chemotherapy and radiofrequency ablation (RFA) for liver metastases.

On Apr. 6th, 2018, she started to receive iNeo-Vac-P01, and underwent a total of 8 doses iNeo-Vac-P01, including 5 prime and 3 boost vaccinations. During treatment, the changes of target lesions were monitored every two months. MRI showed liver lesion regression at 2nd month and 4th month after vaccination compared with baseline level (Fig. 4A). Grade 1 and grade 2 side effects, including chill, fatigue, and muscle soreness were observed and recorded during regimen. No SAE was noted through the whole treatment period. Ex vivo IFN-γ ELISpot of PBMCs showed markedly stronger response (except week 18 due to the poor condition of PBMCs) to all peptide pools at week 3 (average 132.8 spots), week 9 (average 213.9 spots), week 32 (average 87.1 spots) comparing to that of baseline (week 0, average 6.1 spots) (Fig. 4B and C). Notably, pre-treatment PBMCs also showed measurable response to all 5 peptide pools, suggesting that previous radiofrequency ablation might lead to release of tumor neoantigens which further activated specific T cells.

**Discussion**

Patients with various types of advanced solid tumors were enrolled in this study. Despite the significant differences in their tumor mutational burdens (TMBs), for example, those between P009 (pancreatic cancer) and P011 (ovarian cancer) in particular, effective vaccines were designed and prepared for all patients, following the successful identification of patients’ neoantigens by iNeo-Suite. Here, several different vaccine immunization schemes were applied for the patients’ maximum profit and safety, exploring the impact of peptide dosage (100 μg or 300 μg/peptide) and boost immune cycles (1-month or 2-months boost intervals as well as 2 to 6 boost times) on vaccines’ safety and efficacy (Supplementary Table S1). As a result, two cases of acute allergic reaction with similar clinical manifestations occurred in the study, which might result from the increase of boost times, owing to peptide-specific antibody accumulation. This hypothesis needs experimental validation by enzyme-linked immunosorbent assay (ELISA). No significant differences in terms of immunological responses, clinical activities and AEs were observed in this study while applying 100 μg and 300 μg prime doses. However, it’s noticed that patients with more AEs usually had better response, indicating a potential relationship between clinical response and AEs (Supplementary Table S1), which required further validation.

Although none of the enrolled patients was observed with CR or PR, their DCR was relatively high (71.4%, 15/21), with PFS and OS similar to the reported data of NEON Therapeutics Inc. In addition, the values of these indexes could be higher if iRECIST were used instead of standard RECIST v1.1 for our data analysis. So far, both advanced pancreatic cancer and biliary tract
cancer are known for their poor prognosis and short survival times. For example, the first line standard therapy of metastatic pancreatic cancer is nanoliposomal irinotecan plus fluorouracil and folinic acid with median OS of 6.1 months and PFS of 3.1 months (26). However, as of data cutoff, the PFS of our enrolled two pancreatic cancer patients were 4.2 months and 6.3 months respectively, while their OS were 14.0+ months and 13.3+ months respectively. Additionally, the enrolled two biliary tract cancer patients were under deduced stable disease for 10.8+ months and 14.5+ months respectively, potentially longer than the 11.7-months median OS of cisplatin/gemcitabine as the first line standard treatment in advanced biliary cancer (27). And these enrolled advanced pancreatic and biliary tract cancer patients failed to routine line of chemotherapy, immunotherapy or small molecular target therapy. Although these cases cannot stand for a large cohort, the above data suggested that the pancreatic cancer and biliary tract cancer patients in this study might have benefited from our personalized peptide vaccine.

In this study, both the median PFS (4.6 months, 95% CI, 2.5 to 5.2) and OS with a 55.1% estimated percentage at the 12th month (95% CI, 25.9% to 76.9%). Take some typical clinical trials of cancers with high incidence rates for example. In a phase III clinical trial (CheckMate 078) for NSCLC progressed during/after platinum-based doublet chemotherapy, the median OS and PFS of nivolumab (anti-PD-1 antibody) treatment were 12.0 months (95% CI 10.4–14.0) and 2.76 months (95% CI 2.37–3.35) respectively (28). In ATTRACTION-2, a phase III trial for patients with advanced gastric or gastro-esophageal junction cancer failed after at least two previous chemotherapy regimens, the median OS and PFS of nivolumab treatment were 5.26 months (95% CI 4.60-6.37) and 1.61 months (95% CI 1.54–2.30) respectively (29). Additionally, a phase II study of anti-CTLA-4 monoclonal antibody (tremelimumab) for patients with refractory metastatic colorectal cancer demonstrated a median PFS of 2.3 months (95% CI 2.1-2.6) and a median OS of 4.8 months (95% CI 4.1-7.7) (30). Although the sample size was small, the personalized neoantigen vaccine iNeo-Vac-P01 had shown promising anti-tumor efficacy, which needs further study with a larger sample size.

Interestingly, besides patient P008 (Fig. 4B and C), three other patients (P004, P014, P019) out of the 9 post-RFA patients (44%) also displayed evident neoantigen-specific T cell response before vaccination, while there was only 1 out of 13 patients (8%) in non-RFA group found having pre-vaccination response (Fig. 1B and Supplementary Table S10). It is probably due to the fact that, different from complete surgical resection, RFA treatment can result in tumor necrosis, which will become an immunogenic source, providing pro-inflammatory signals (31,32). After RFA, it may activate and/or generate a large amount of IFN-α and/or neoantigen, while improving the expression of other co-stimulating factors and the presenting of tumor antigen to T cells. Additionally, the pre-vaccination responses seemed unrelated with tumor type, position of RFA, or time interval between last RFA and vaccination (Supplementary Table S10). The iNeo-Vac-P01 vaccination post RFA could form an effective anti-tumor T-cell response, which worth further study.

Furthermore, compared with non-RFA group, RFA group seemed to have a longer survival (unpublished data). Similarly, in the case study of patient 005 who received iNeo-Vac-P01 after PD-1 antibody treatment, we noticed that the combinatorial therapies of PD-1/L1 antibodies and personalized neoantigen vaccine may be able to provide better anti-cancer therapeutic effects. More work needs to be done to evaluate the potential benefits from the combinatorial therapies of
iNeo-Vac-P01 with RFA or PD1/L1 antibodies.

In addition, we also identified several potential biomarkers for clinical response, which required further validation. For example, the mutations of gene MUC, ZFHX3 and ABL1 seemed to predict poor response, whereas the copy number variations of gene TNFRSF, SOX3 and MAGE were relatively associated with better response. Meanwhile, the increase of peripheral T cells during treatment implied patients’ better response. More patients would be enrolled in future study to validate our hypothesis.

In general, the preliminary results demonstrated the feasibility, safety and efficacy of iNeo-Vac-P01 on patients with various types of advanced solid tumors. iNeo-Vac-P01 monotherapy could elicit robust neoantigen-specific T cell response, significantly increase T cell infiltration into tumors and remodel tumor-immune microenvironment alone, suggesting its great potential as cancer immunotherapy. Until now, 22 patients have participated in this clinical trial, more than all of the previous reported patient numbers that we found in the similar kind of clinical trials. In future, we plan to enroll more patients with early- and intermediate-stage cancers, including those who require recurrence prevention after surgery, making further efforts to explore the anti-tumor and anti-recurrence efficacy of iNeo-Vac-P01. And the combination of iNeo-Vac-P01 with RFA and/or anti-PD-1/L1 antibody is worth further exploring in the future for advanced cancer patient, especially oligometastatic patients.

Acknowledgments

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References


Tables:

Table 1. Demographic and disease characteristics at baseline

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Patients (N=22)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age-yr.</strong></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>59±10</td>
</tr>
<tr>
<td>Range</td>
<td>28-79</td>
</tr>
<tr>
<td><strong>Age category-no. (%)</strong></td>
<td></td>
</tr>
<tr>
<td>&lt;65 yrs.</td>
<td>16 (72.73)</td>
</tr>
<tr>
<td>≥65 yrs.</td>
<td>6 (27.27)</td>
</tr>
<tr>
<td><strong>Sex-no. (%)</strong></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>12 (54.55)</td>
</tr>
<tr>
<td>Female</td>
<td>10 (45.45)</td>
</tr>
<tr>
<td><strong>Metastatic sites-no. (%)</strong></td>
<td></td>
</tr>
<tr>
<td>Liver</td>
<td>5 (22.72%)</td>
</tr>
<tr>
<td>Lung</td>
<td>4 (18.18%)</td>
</tr>
<tr>
<td>Both liver and lung</td>
<td>6 (27.27%)</td>
</tr>
<tr>
<td>Bone</td>
<td>4 (18.18%)</td>
</tr>
</tbody>
</table>
| **ECOG performance-status score-no. (%)** | |*
| 0                             | 7 (31.82)       |
| 1                             | 15 (68.18)      |
| **Radiotherapy-no. (%)**      |                 |
| Yes                           | 11 (50.00)      |
| No                            | 11 (50.00)      |
| **Lines of prior systematic therapy-no. (%)** | | |
| 2                             | 7 (31.82)       |
| ≥3                            | 15 (68.18)      |
| **Tumor type-no. (%)**        |                 |
| Melanoma                      | 4 (18.18)       |
| Colon Cancer                  | 4 (18.18)       |
| Non-Small Cell Lung Cancer    | 3 (13.64)       |
| Pancreatic Cancer             | 2 (9.09)        |
| Biliary Tract Cancer          | 2 (9.09)        |
| Ovarian Cancer                | 2 (9.09)        |
| Others                        | 5 (22.73)       |

*Eastern Cooperative Oncology Group (ECOG) performance-status scores range from 0 to 5, with 0 indicating no symptoms and higher scores indicating increasing disability.
Table 2. Treatment related AEs in all treated patients*

<table>
<thead>
<tr>
<th></th>
<th>Any Grade</th>
<th></th>
<th>Grades 3 to 4</th>
<th></th>
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<tr>
<td></td>
<td>No.</td>
<td>%</td>
<td>No.</td>
<td>%</td>
</tr>
<tr>
<td>Any AE</td>
<td>12</td>
<td>54.55</td>
<td>2</td>
<td>9.09</td>
</tr>
<tr>
<td>Fatigue</td>
<td>8</td>
<td>36.36</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Chill</td>
<td>4</td>
<td>18.18</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Fever</td>
<td>3</td>
<td>13.64</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Emesis</td>
<td>2</td>
<td>9.09</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Muscle soreness</td>
<td>2</td>
<td>9.09</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Injection site reaction</td>
<td>1</td>
<td>4.55</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Dizzy</td>
<td>1</td>
<td>4.55</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Nausea</td>
<td>1</td>
<td>4.55</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Upper gastrointestinal hemorrhage</td>
<td>1</td>
<td>4.55</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Lose weight</td>
<td>1</td>
<td>4.55</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Acute allergic reaction</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>9.09</td>
</tr>
</tbody>
</table>

*Including all patients who received at least one dose of a trial treatment. Events were attributed to treatment by the investigator and are listed as indicated by the investigator on the case-report form.
Figure Legends:

Fig. 1. *iNeo-Vac-P01 induced specific T cell response and anti-tumor activation.* (A) Swimmer plot showed the follow-up information of enrolled patients (N=22) in a time range of 16 months. Light gray lines and yellow dots indicated the period of prime and the time points of boost vaccinations respectively. The depiction of disease condition and patient status were indicated by lines and dots with various colors. Patient numbers were shown as the values of y-axis. (B) For each patient marked in X-axis, green triangle and red diamond represented the relative response rates, which are equal to the ratios of peptides (or peptide pools for patients signed with “#”) with positive ELISpot results to all immunized peptides (or peptide pools for patients signed with “#”) before and after vaccination respectively. Asterisks indicated there were new high abundant or clones with considerably increased abundance detected in peripheral T cells of particular patients after vaccination. The bar chart with secondary Y-axis represented the IFN-γ spots per 10^5 PBMCs of the peptide or peptide pool with best response for each patient. (C) The multiplexed IF images of FFPE samples obtained from patient P008, P013 and P015’s pre- and post-vaccination. CD8 (red in P008 and P013, pink in P015) and Granzyme B (yellow) double positive T cells, CD4 (green) and Granzyme B (yellow) double positive T cells, and the merged signals were showed on the left. The proportion (%) and density (counts per mm^2) of the CD8 and Granzyme B double positive T cells and CD4 and Granzyme B double positive T cells were shown on the right.

Fig. 2. *Clinical response induced by iNeo-Vac-P01.* (A) Percentage changes of tumor lesion size from baseline were recorded over a period of 55 weeks. (B) The waterfall plots recorded the best clinical response of patients enrolled. Dashed lines above and below indicate 20% increase or 30% reduction of the sum of the longest diameter of the tumor respectively, which are in accordance with the cut-off value for progressive disease and partial response by RECIST 1.1. And the P005 patient was diagnosed as pseudo-progression as the change of target tumor lesion change. (C and D) Figures showed the Kaplan–Meier survival curves with PFS (C) and OS (D). Tick marks represented data censored as at the last time that the patient was known to be (C) stable disease or unknown and (D) alive.

Fig. 3. *A case report of Patient 005, iNeo-Vac-P01 mediated specific T cell response and tumor regression.* (A) Figure showed the treatment scheme of patient 005. (B) CT scan images showed the series changes of left upper lung lesion (upper panel) and left lower lung lesion (bottom panel) during the treatment. The red arrows indicated the correspondingly tumor lesions. (C) and (D) Ex vivo IFN-γ and ELISpot of PBMCs was performed with peptides at different time points. The dimethyl sulfoxide (DMSO) was used as negative control and mixed peptides from CEF (including peptides of cytomegalovirus, epstein–barr virus and influenza virus) were used as positive control. (E) Increased abundance of peripheral T cell clones after vaccination was detected by TCR sequencing.

Fig. 4. *A case report of Patient 008, iNeo-Vac-P01 mediated specific T cell response and tumor regression.* (A) MRI scan was performed at baseline and approximately 2 months, 4 months after vaccination. The red arrows indicated the target tumor lesions. (B) and (C) Ex vivo IFN-γ ELISpot of PBMCs was performed with vaccine peptides at different time points. DMSO was the negative control and CEF was the positive control.
**Figure 1**

A. A diagram showing the timeline of treatment initiation and events such as Prime vaccine, Boost vaccine, Stable disease, Progressive disease, Unknown disease, Study drop-out, and Death.

B. A graph showing the response rate (%) and IFN-γ spot counts with pre-vax and post-vax data points labeled with symbols #P001 to P022.

C. Images illustrating CD8+ GZB+ and CD4+ GZB+ T cells with pre-vax and post-vax conditions for P008, P013, and P015.

* Relatively high abundant novel clones of peripheral T cells detected after vaccination

# ELISpot assay performed with peptide pools

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Figure 2

A. Change of target lesions from baseline (%)

B. Best Clinical Response (%)

C. Progression free survival (%)

D. Overall survival (%)

Legend:
- Pancreatic Carcinoma
- Colon Cancer
- Biliary Tract Cancer
- Lung Cancer
- Melanoma
- Others

Key:
- First occurrence of new lesion
- Non-target lesion progression
- % change truncated to 100%

Graphs show data for different types of cancers and their progression over time.
Figure 3

A) Timeline of vaccine administration and tumor progression/regression.

B) Images showing tumor progression over time.

C) Graph showing IFN-γ spots per 10^5 PBMCs for different genes.

D) Heatmap showing gene expression changes over time.

E) Line graph showing abundance of peripheral T cells before and after treatment.
Figure 4

A

Baseline 2nd month 5th month

B

IFN-γ spots per 10^5 PBMCs

Week 0 Week 3 Week 9 Week 18* Week 32

Pool 1 Pool 2 Pool 3 Pool 4 Pool 5

*The PBMCs of week 18 in poor condition

C

Week 0 Week 3 Week 9 Week 18* Week 32

CEF DMSO Pool 1 Pool 2 Pool 3 Pool 4 Pool 5

*The PBMCs of week 18 in poor condition
A pan-cancer clinical study of personalized neoantigen vaccine monotherapy in treating patients with various types of advanced solid tumors

Yong Fang, Fan Mo, Jiawei Shou, et al.

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