

Quantitative analysis and clonal characterization of T-cell receptor β repertoires in patients with advanced non-small cell lung cancer treated with cancer vaccine

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Abstract. With the development of cancer immunotherapy that may activate T cells, a practical and quantitative method to improve monitoring and/or prediction of immunological response of patients as a predictive biomarker is of importance. To examine possible biomarkers for a therapeutic cancer vaccine containing a mixture of three epitope peptides derived from cell division-associated 1, lymphocyte antigen 6 complex locus K and insulin-like growth factor-II mRNA-binding protein 3, T-cell receptor β (TCR β) repertoires of blood samples from 24 patients with human leukocyte antigen-A*2402-positive non-small cell lung cancer were characterized prior to and

following 8 weeks of the cancer vaccine treatment, by applying a next-generation sequencing method. It was identified that 14 patients with overall survival (OS) times of ≥ 12 months had significantly lower TCR β diversity indexes in samples prior to treatment, compared with 10 patients who succumbed within 1 year ($P=0.03$). In addition, patients with a high level of activated CD8⁺ T cells that are defined by a high granzyme A/CD8 ratio had favorable OS rates (log-rank test, $P=0.04$). The TCR β diversity index and immunogenic gene markers following vaccine administration may serve as predictive or monitoring biomarkers for cancer vaccine treatment.

Introduction

Despite increasing knowledge about lung cancer and its treatment modalities over the last few decades, this disease continues to be responsible for the largest number of mortalities in males and females worldwide (1). Lung cancer was estimated to account for 27% of all cancer-associated mortalities in 2014 (2). There are two major histological types of lung cancer: Small cell lung cancer (SCLC) and non-SCLC (NSCLC) (3). Standard treatments for NSCLC include combinations of surgery, chemotherapy and radiotherapy (4). Although most advanced-stage patients receive chemotherapy and achieve clinical responses to a certain extent, the majority eventually experience relapse (5,6). The 5-year survival rate of patients with NSCLC only marginally improved from 15.9 in 2008 to 18.0% in 2014 (2,7,8). Therefore, novel therapies are required to ensure improved management of NSCLC.

Immunotherapy is a novel therapeutic strategy that is currently being evaluated for the treatment of NSCLC (4). Until recently, studies of vaccine treatment for patients with lung cancer have not yielded very positive results either due to non-specific immune system activation or due to toxicity (9-13). The lung, which has a high level of

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Abbreviations: NSCLC, non-small-cell lung cancer; TCR, T-cell receptor; HLA, human leukocyte antigen; CDR3, complementary-determining region 3; PBMC, peripheral blood mononuclear cell; PBL, peripheral blood lymphocyte; DI, diversity index

Key words: lung cancer, non-small cell lung cancer, cancer vaccine, T-cell receptor, next-generation sequencing, diversity index, immunogenicity

environmental exposure, has long been considered a poorly immunogenic tumor site (14). Therefore, an effective vaccine against NSCLC should contain antigens that are specific to the tumor cells and have the ability to generate immunogenicity following administration (15). Previously, cDNA microarray and laser microdissection were used to identify three genes for which transcripts were observed at high levels in cells from lung cancer, esophageal cancer, testis and placenta, but not in normal cells: Cell division-associated 1 (CDCA1) (16,17), lymphocyte antigen 6 complex locus K (LY6K) (17,18) and insulin-like growth factor-II mRNA-binding protein 3 (IMP-3) (17,19). Accordingly, a vaccine comprising three human leukocyte antigen (HLA)-A24-restricted epitope peptides derived from these genes was developed for NSCLC and esophageal cancer (20). A Phase I clinical study of a combination of three peptides, including LY6K and IMP-3, in patients with advanced esophageal squamous cell carcinoma demonstrated that the vaccine was well tolerated and that strong T-cell responses to these specific antigens were induced following vaccination (21). Previously, a Phase II clinical study of a combination of three peptides (LY6K, IMP-3 and CDCA1) in patients with advanced head and neck squamous cell carcinoma also reported peptide-specific cytotoxic T lymphocyte (CTL) responses in the majority of the HLA-A2402-positive patients (22). In the present study, a Phase II clinical trial with exploratory investigations was conducted using a vaccine comprising these three peptides in patients with advanced NSCLC who were refractory to standard therapies (15).

One important predictive biomarker of vaccine therapy efficacy is the ability to induce an immunogenic response against specific cancer cells (23). Traditionally, enzyme-linked immune-spot and HLA-multimer assays have been used to measure CTL responses, since a high level of CTL infiltration into a tumor was reported to be associated with a good response to treatment (24). However, these assays require the *ex vivo* expansion of peripheral blood lymphocytes (PBLs) via stimulation and are not practical in clinical settings (25). In addition, lung tumor biopsy to examine intratumoral CTL infiltration is invasive (26). Therefore, it may be preferable to identify biomarkers from easily available human materials, including blood, using the predictive power that has been demonstrated to associate well with overall survival (OS) rate (27). The expansion and activation of certain T-cell populations, including cytotoxic CD8⁺ T cells, has been reported to be beneficial for the recognition and elimination of cancer cells (28,29). By contrast, the expansion of regulatory T cells may be harmful, as these T cells protect cancer cells by suppressing tumor-specific CD8⁺ cytotoxic T cells (30-33). Therefore, it is important to quantitatively characterize the T-cell receptor (TCR) repertoires of patients with cancer prior to and following immunotherapy, including cancer vaccine treatment, to improve the understanding of the molecular mechanism underlying treatment efficacy.

In total ~95% of T cells express TCR, which is a heterodimer of the TCR α and TCR β chains, a signature of each T lymphocyte (34). To date, the TCR α gene on chromosome 14 has been reported to comprise a total of 70 variable (V) exons, 61 joining (J) exons and 1 constant (C) exon, whereas the TCR β gene on chromosome 7 comprises 60 V exons, 15 J exons and 2 C exons (35). In addition, TCR β contains

two diversity (D) exons; accordingly, the TCR genes undergo somatic V(D)J recombination, resulting in a significant increase in TCR diversity (36-38). This V(D)J segment rearrangement results in the highly variable complementary-determining region (CDR3), thus allowing the recognition of any possible antigens presented by HLA molecules (39). It has been estimated that ~10¹⁸ different TCRs are generated in humans (25).

Advances in next-generation sequencing technology have made it possible to sequence millions of TCR cDNAs, and thus characterize patient TCR repertoires in a single experiment (40-42). In the present study, it was hypothesized that the expansion and activation of a large number of T-cell populations, particularly the cytotoxic T-cell population, may be used as predictive biomarkers in response to vaccine treatment to assess the outcomes of patients with NSCLC who had received cancer vaccine therapy. In the present study, T-cell repertoires and certain immune-associated molecules in patients with advanced NSCLC with an HLA-A*2402 who received the cancer vaccine treatment were characterized using cDNA-sequencing technology and a gene expression assay.

Materials and methods

Vaccines and patients. A total of 53 patients with advanced NSCLC resistant to standard therapies were enrolled between 21 May 2012 and 4 April 2013 in a Phase II open-label multicenter non-randomized clinical cancer vaccination trial conducted in an exploratory setting. Patients were vaccinated with a mixture of 1 mg each of three HLA-A24-restricted epitope peptides derived from CDCA1, LY6K and IMP-3 mixed with incomplete Freund's adjuvant (Montanide ISA 51; SEPPIC, Puteaux, France) (trial no. NCT01592617). The clinical characteristics and treatment information for all 53 patients enrolled in the clinical trial are summarized in Table I. Patients received weekly subcutaneous injections of the peptides into the axillary region until disease progression was observed or the patient declined continued vaccine treatment. Written informed consent was obtained from all individuals enrolled in the trial. The trials were carried out in accordance with The Declaration of Helsinki on experimentation on human subjects, under the approval of the institutional ethics committees of the individual institutes. TCR β sequencing and gene expression analysis were performed for blood samples from 24/35 HLA-A*2402-positive patients obtained.

Peripheral blood mononuclear cell collection. Blood samples were collected from patients prior to and following 8 weeks of vaccine treatment. Blood was drawn into BD Vacutainer® CPT™ cell preparation tubes containing sodium citrate (CPT; BD Biosciences, Franklin Lakes, NJ, USA). Samples were immediately centrifuged at 400 x g for 20 min at room temperature. Peripheral blood mononuclear cells (PBMCs) were collected from the second layer of the column and washed with PBS. Cell numbers were determined using a hemocytometer, and cell viability was assessed via trypan blue staining. Following treatment with trypan blue, cells inside the four large corner squares were counted at x100 magnification under the light microscope (CKX41; Olympus Corporation, Tokyo, Japan). A total of ~8x10⁶ viable cells/sample were used for RNA isolation.

RNA isolation and polymerase chain reaction (PCR) amplification. An RNeasy mini kit (Qiagen, Inc., Valencia, CA, USA) was used to isolate total RNA from PBMCs. In total 10 μ l DNase I (from the RNeasy mini kit) treatment was applied to remove undesirable genomic DNA contamination. A SMART cDNA library construction kit (Clontech Laboratories, Inc., Mountain View, CA, USA) was used to synthesize cDNA with a common adaptor (SMART IV oligonucleotide) at the 5'-ends. PCRs were performed to amplify TCR β cDNAs. All possible TCR β combinations were captured using a common forward primer (5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGTATCAACGCAGAGTGGCCAT-3') complementary to the SMART IV adaptor and a reverse primer specific for the constant region (5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGDVHDTVCTGATGGCTCAAACACAGC-3'). The PCR protocol was as follows: 94°C for 3 min; followed by 30 cycles of 94°C for 30 sec, 65°C for 30 sec and 72°C for 1 min. Size selection (between 300 and 950 base pairs) was conducted using Pippin Prep (Sage Science, Beverly, MA, USA) to collect products known to be of the expected size (43). This experiment was performed once with the patient blood sample.

Library preparation and sequencing. A Nextera XT DNA library kit (Illumina, Inc., San Diego, CA, USA) was used to add adapter sequences onto template DNA to generate multiplexed sequencing libraries, allowing the sequencing and distinction of multiple samples in a single experiment. The PCR protocol was as follows: 95°C for 3 min; 8 cycles of 95°C for 30 sec, 55°C for 30 sec and 72°C for 30 sec; and a hold step at 72°C for 5 min. Multiple dual index-encoded samples were combined in a single sequencing library. This library was loaded onto the MiSeq Reagent kit (version 3; Illumina, Inc.) and sequenced using an Illumina MiSeq Desktop Sequencer (Illumina, Inc.).

TCR sequence analysis. FASTQ files containing sequencing reads were generated using the MiSeq sequencer and mapped to the reference sequences derived from IMGT/GENE-DB (www.imgt.org) with the Bowtie2 alignment program (version 2.1.0; <http://bowtie-bio.sourceforge.net/bowtie2/index.shtml>) (44). To determine CDR3 in TCR β , a conserved cysteine residue encoded in the 3' end of the V segment and a conserved phenylalanine residue encoded in the 5' end of the J segment, which signal CDR3, were identified as described previously (25). Amino acid sequences were determined using the nucleotide sequences between the conserved TCR V cysteine residue and TCR J phenylalanine residue.

Gene expression assay. First-strand cDNA products as aforementioned were used in a gene expression assay to analyze the expression of CD4, CD8 and granzyme A (GZMA) transcripts. A PCR TaqMan gene expression assay was performed on an ABI ViiA™ 7 system (Applied Biosystems; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. All CD4, CD8 and GZMA transcript expression levels were normalized to the transcript expression of the housekeeping gene GAPDH.

Statistical analysis. OS rates were analyzed using the Kaplan-Meier estimator method, and survival was measured

in days between the first vaccination and mortality. Progression-free survival (PFS) was measured in days from the first vaccination to the date of first documented disease progression or the date of mortality from any cause. The statistical significance of the survival period was analyzed using the Harrington-Fleming test.

The diversity indexes (DIs) of CDR3 sequences were calculated using inverse Simpson's index formula as previously reported (25). The DI reflects the total number and also the evenness of the identified clonotypes.

The Mann-Whitney test was used to compare the DIs between the long-survival and short-survival groups. A paired Student's *t*-test was performed to compare the DI prior to and following vaccine treatment within each group. These statistical tests were conducted using Prism software (version 6.0; GraphPad, Inc., La Jolla, CA, USA). The median was used as a threshold point to divide into two groups: High GZMA/CD8 and low GZMA/CD8 or high CD8/CD4 and low CD8/CD4. A log-rank test was performed using R software (version 3.2.0; The R Project for Statistical Computing, Vienna, Austria) to compare the percentage survival in these two groups. Data are presented as the mean \pm standard error. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Association between the TCR β DI and survival status of patients with NSCLC treated with cancer peptide vaccines. Patients with NSCLC (35 HLA-A*2402-positive and 18 others) who had previously failed standard therapies were recruited, as summarized in Table I. The median OS time of these patients was 400 days, whereas the PFS was 57 days. No significant difference in the clinical outcomes between the HLA-A*2402-positive and -negative groups was identified. None of the patients exhibited a complete response according to the Response Evaluation Criteria in Solid Tumors (45). The most common adverse events of any grades observed during the present study are described in Table II.

Blood samples from 24 of 35 HLA-A*2402-positive patients were obtained, 14 of which achieved stable disease and/or remained alive >12 months after enrollment, and possible predictive immune biomarkers, including TCR β analysis, were examined. The TCR β DIs were compared between long-term survivors (lived for >12 months after enrollment) and short-term survivors (died within 12 months of enrollment) by calculating an inverse Simpson's DI (1/D) of the TCR β repertoire, as described in the Materials and methods section. The present data revealed that prior to vaccine treatment, patients in the long-term survival group exhibited significantly lower TCR β DIs compared with short-term survivors (63 ± 61 vs. 259 ± 307 ; $P = 0.03$; Fig. 1A). However, the difference between the two groups was not significant in blood samples following 8 weeks of treatment (107 ± 108 vs. 188 ± 165 ; $P = 0.12$; Fig. 1B) although a trend in the increase in DI was observed in the long-term survival group (mean of differences, 44 ± 7 ; $P = 0.07$; Fig. 1C). By contrast, no significant change in DI between samples prior to and following the treatment was observed in the short-term survival group (Fig. 1D). The difference in the changes in DI in the long-term and short-term groups is presented in Fig. 1E.

Table I. Background of patients.

Characteristics	Total, n	HLA-A*2402(+), n	HLA-A*2402(-), n
Total	53	35	18
Median age \pm SD, years	64.0 \pm 7.7	64.1 \pm 7.5	63.9 \pm 8.2
Sex			
Female	21	16	5
Male	32	19	13
Location of primary lesion			
Pulmonary hilum	5	5	0
Lung field	43	28	15
Pleural effusion	1	0	1
Missing	4	2	2
Histological type			
Adenocarcinoma	43	28	15
Squamous cell carcinoma	9	7	2
Pleomorphic carcinoma	1	0	1
T factor			
TX	19	9	10
T0	2	1	1
T1a	3	3	0
T1b	1	1	0
T2a	4	4	0
T2b	1	0	1
T3	7	6	1
T4	16	11	5
N factor			
NX	4	2	2
N0	18	11	7
N1	2	2	0
N2	18	14	4
N3	11	6	5
M factor			
MX	0	0	0
M0	9	8	1
M1a	20	13	7
M1b	17	9	8
M1a+1b	7	5	2
ECOG performance status score			
0	38	25	13
1	11	8	3
2	4	2	2
Smoking status			
Current or former smoker	30	20	10
Never smoked	23	15	8
EGFR mutation status			
Positive	13	6	7
Not detected	36	27	9
Not reported	4	2	2

HLA, human leukocyte antigen; EGFR, epidermal growth factor receptor; T, tumor; N, node; M, metastasis; ECOG, Eastern Cooperative Oncology Group.

Table II. Treatment-associated adverse effects.

Adverse events	HLA-A*2402(+) (n=35)		HLA-A*2402(-) (n=18)		Total (n=53)	
	Cases (incidence rate), n (%)	Events, n	Cases (incidence rate), n (%)	Events, n	Cases (incidence rate), n (%)	Events, n
Total	34 (97.1)	159	16 (88.9)	56	51 (94.3)	215
Infectious disease	13 (37.1)	20	3 (16.7)	4	16 (30.2)	24
Nasopharyngitis	6 (17.1)	7	1 (5.6)	1	7 (13.2)	8
Herpes zoster	2 (5.7)	2	0 (0.0)	0	2 (3.8)	2
Periodontitis	1 (2.9)	1	1 (5.6)	1	2 (3.8)	2
Bronchitis	1 (2.9)	1	0 (0.0)	0	1 (1.9)	1
Cellulitis	1 (2.9)	1	0 (0.0)	0	1 (1.9)	1
Influenza	1 (2.9)	1	0 (0.0)	0	1 (1.9)	1
Esophageal candidiasis	1 (2.9)	1	0 (0.0)	0	1 (1.9)	1
Paronychia	0 (0.0)	0	1 (5.6)	1	1 (1.9)	1
Pneumonitis	0 (0.0)	0	1 (5.6)	1	1 (1.9)	1
Pulpitis	1 (2.9)	1	0 (0.0)	0	1 (1.9)	1
Rhinitis	1 (2.9)	1	0 (0.0)	0	1 (1.9)	1
Foot tinea pedis	1 (2.9)	1	0 (0.0)	0	1 (1.9)	1
Tonsillitis	1 (2.9)	1	0 (0.0)	0	1 (1.9)	1
Urinary tract infection	1 (2.9)	1	0 (0.0)	0	1 (1.9)	1
Device related infection	1 (2.9)	1	0 (0.0)	0	1 (1.9)	1
Malignant neoplasm	9 (25.7)	9	3 (16.7)	4	12 (22.6)	13
Malignant pleuritis	2 (5.7)	2	2 (11.1)	2	4 (7.5)	4
Central nervous system metastasis	2 (5.7)	2	0 (0.0)	0	2 (3.8)	2
NSCLC	2 (5.7)	2	0 (0.0)	0	2 (3.8)	2
Malignant ascites	1 (2.9)	1	0 (0.0)	0	1 (1.9)	1
Esophageal cancer	1 (2.9)	1	0 (0.0)	0	1 (1.9)	1
Tumor infiltration of bone marrow	1 (2.9)	1	0 (0.0)	0	1 (1.9)	1
Meningeal metastasis	0 (0.0)	0	1 (5.6)	1	1 (1.9)	1
Brain tumor	0 (0.0)	0	1 (5.6)	1	1 (1.9)	1
Metabolic disease	2 (5.7)	2	0 (0.0)	0	2 (3.8)	2
Hyperuricemia	1 (2.9)	1	0 (0.0)	0	1 (1.9)	1
Hyperlipidemia	1 (2.9)	1	0 (0.0)	0	1 (1.9)	1
Mental disease	1 (2.9)	1	1 (5.6)	1	2 (3.8)	2
Insomnia	1 (2.9)	1	1 (5.6)	1	2 (3.8)	2
Nervous system disorder	6 (17.1)	8	1 (5.6)	1	7 (13.2)	9
Headache	3 (8.6)	3	0 (0.0)	0	3 (5.7)	3
Brain compression	1 (2.9)	1	0 (0.0)	0	1 (1.9)	1
Convulsion	1 (2.9)	2	0 (0.0)	0	1 (1.9)	2
Dizziness	1 (2.9)	1	0 (0.0)	0	1 (1.9)	1
Hypoesthesia	0 (0.0)	0	1 (5.6)	1	1 (1.9)	1
Drowsiness	1 (2.9)	1	0 (0.0)	0	1 (1.9)	1
Eye disease	1 (2.9)	1	1 (5.6)	1	2 (3.8)	2
Cataract	1 (2.9)	1	0 (0.0)	0	1 (1.9)	1
Dry eye	0 (0.0)	0	1 (5.6)	1	1 (1.9)	1
Ear disease and labyrinthine disturbance	1 (2.9)	1	0 (0.0)	0	1 (1.9)	1
Rotary vertigo	1 (2.9)	1	0 (0.0)	0	1 (1.9)	1
Heart disease	2 (5.7)	4	0 (0.0)	0	2 (3.8)	4
Arrhythmia	1 (2.9)	1	0 (0.0)	0	1 (1.9)	1
Pericardial effusion collection	1 (2.9)	1	0 (0.0)	0	1 (1.9)	1
Angina	1 (2.9)	1	0 (0.0)	0	1 (1.9)	1

Table II. Continued.

Adverse events	HLA-A*2402(+) (n=35)		HLA-A*2402(-) (n=18)		Total (n=53)	
	Cases (incidence rate), n (%)	Events, n	Cases (incidence rate), n (%)	Events, n	Cases (incidence rate), n (%)	Events, n
Supraventricular tachycardia	1 (2.9)	1	0 (0.0)	0	1 (1.9)	1
Angiopathy	0 (0.0)	0	1 (5.6)	1	1 (1.9)	1
Hypertension	0 (0.0)	0	1 (5.6)	1	1 (1.9)	1
Respiratory disease	15 (42.9)	18	4 (22.2)	7	19 (35.8)	25
Upper respiratory infection	11 (31.4)	13	2 (11.1)	4	13 (24.5)	17
Allergic rhinitis	1 (2.9)	1	1 (5.6)	1	2 (3.8)	2
Atelectasis	0 (0.0)	0	1 (5.6)	1	1 (1.9)	1
Voice disturbance	1 (2.9)	1	0 (0.0)	0	1 (1.9)	1
Dyspnea	0 (0.0)	0	1 (5.6)	1	1 (1.9)	1
Pleuritis	1 (2.9)	1	0 (0.0)	0	1 (1.9)	1
Sneezing	1 (2.9)	1	0 (0.0)	0	1 (1.9)	1
Hypertrophic rhinitis	1 (2.9)	1	0 (0.0)	0	1 (1.9)	1
Gastrointestinal injury	13 (37.1)	15	5 (27.8)	7	18 (34.0)	22
Constipation	5 (14.3)	5	2 (11.1)	2	7 (13.2)	7
Diarrhea	3 (8.6)	3	0 (0.0)	0	3 (5.7)	3
Vomiting	1 (2.9)	1	2 (11.1)	3	3 (5.7)	4
Nausea	2 (5.7)	2	0 (0.0)	0	2 (3.8)	2
Abdominal discomfort	1 (2.9)	1	0 (0.0)	0	1 (1.9)	1
Enteritis	0 (0.0)	0	1 (5.6)	1	1 (1.9)	1
Gastritis	1 (2.9)	1	0 (0.0)	0	1 (1.9)	1
Hemorrhoid	0 (0.0)	0	1 (5.6)	1	1 (1.9)	1
Esophageal stenosis	1 (2.9)	1	0 (0.0)	0	1 (1.9)	1
Stomatitis	1 (2.9)	1	0 (0.0)	0	1 (1.9)	1
Biliary system disorders	1 (2.9)	1	0 (0.0)	0	1 (1.9)	1
Cholecystitis	1 (2.9)	1	0 (0.0)	0	1 (1.9)	1
Skin disease	7 (20.0)	9	1 (5.6)	1	8 (15.1)	10
Rash	2 (5.7)	2	0 (0.0)	0	2 (3.8)	2
Alopecia	0 (0.0)	0	1 (5.6)	1	1 (1.9)	1
Xerosis cutis	1 (2.9)	1	0 (0.0)	0	1 (1.9)	1
Asteatotic eczema	1 (2.9)	1	0 (0.0)	0	1 (1.9)	1
Erythema	1 (2.9)	3	0 (0.0)	0	1 (1.9)	3
Skeletal muscle or soft tissue disorder	5 (14.3)	5	1 (5.6)	2	6 (11.3)	7
Back pain	1 (2.9)	1	0 (0.0)	0	1 (1.9)	1
Fasciitis	0 (0.0)	0	1 (5.6)	2	1 (1.9)	2
Melalgia	1 (2.9)	1	0 (0.0)	0	1 (1.9)	1
Periarthritis	1 (2.9)	1	0 (0.0)	0	1 (1.9)	1
Spondylosis deformans	1 (2.9)	1	0 (0.0)	0	1 (1.9)	1
Synovial cyst	1 (2.9)	1	0 (0.0)	0	1 (1.9)	1
Kidney or urinary disorder	0 (0.0)	0	1 (5.6)	1	1 (1.9)	1
Strangury	0 (0.0)	0	1 (5.6)	1	1 (1.9)	1
Body or injection site disorder	34 (97.1)	51	14 (77.8)	22	48 (90.6)	73
Injection site reaction	33 (94.3)	41	13 (72.2)	20	46 (86.8)	61
High fever	2 (5.7)	5	1 (5.6)	1	3 (5.7)	6
Disease progression	2 (5.7)	2	1 (5.6)	1	3 (5.7)	3
General fatigue	1 (2.9)	1	0 (0.0)	0	1 (1.9)	1
Injection site bleeding	1 (2.9)	1	0 (0.0)	0	1 (1.9)	1
Pain	1 (2.9)	1	0 (0.0)	0	1 (1.9)	1

Table II. Continued.

Adverse events	HLA-A*2402(+) (n=35)		HLA-A*2402(-) (n=18)		Total (n=53)	
	Cases (incidence rate), n (%)	Events, n	Cases (incidence rate), n (%)	Events, n	Cases (incidence rate), n (%)	Events, n
Laboratory examination	7 (20.0)	9	2 (11.1)	3	9 (17.0)	12
Elevated GTP γ	2 (5.7)	2	1 (5.6)	1	3 (5.7)	3
Lymphocytopenia	2 (5.7)	2	0 (0.0)	0	2 (3.8)	2
Elevated hepatic transaminase	0 (0.0)	0	1 (5.6)	1	1 (1.9)	1
Elevated CPK	1 (2.9)	1	0 (0.0)	0	1 (1.9)	1
Elevated serum potassium level	0 (0.0)	0	1 (5.6)	1	1 (1.9)	1
Elevated hepatic transaminase	0 (0.0)	0	1 (5.6)	1	1 (1.9)	1
Increases in urine glucose levels	1 (2.9)	1	0 (0.0)	0	1 (1.9)	1
Abnormal liver function test	1 (2.9)	1	0 (0.0)	0	1 (1.9)	1
Albuminuria	1 (2.9)	1	0 (0.0)	0	1 (1.9)	1
Weight loss	1 (2.9)	1	0 (0.0)	0	1 (1.9)	1
Injury, toxicosis and procedural complication	5 (14.3)	5	1 (5.6)	1	6 (11.3)	6
Fractured sacrum	0 (0.0)	0	1 (5.6)	1	1 (1.9)	1
Lacerated wound	1 (2.9)	1	0 (0.0)	0	1 (1.9)	1
Ligament sprain	1 (2.9)	1	0 (0.0)	0	1 (1.9)	1
Ecchymoma	1 (2.9)	1	0 (0.0)	0	1 (1.9)	1
Bruise	1 (2.9)	1	0 (0.0)	0	1 (1.9)	1
Radiation dermatitis	1 (2.9)	1	0 (0.0)	0	1 (1.9)	1

HLA, human leukocyte antigen; CPK, creatine phosphokinase; NSCLC, non-small cell lung cancer.

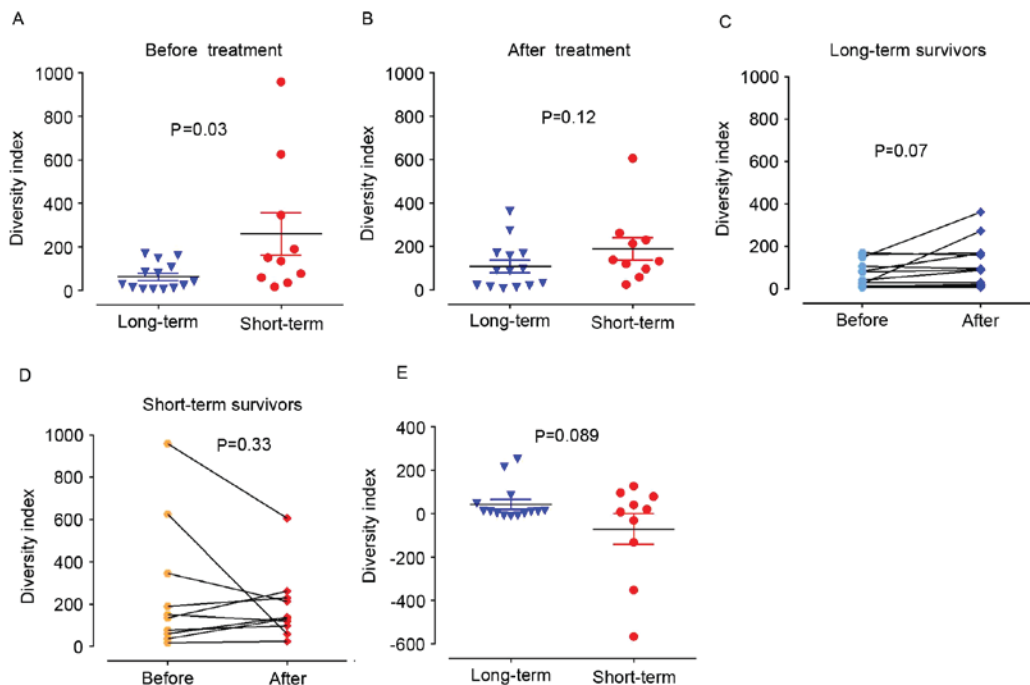


Figure 1. TCR β diversity index analysis. (A) Diversity index prior to treatment. (B) Diversity index following treatment. Blue: Long-term survival group (n=14). Red: Short-term survival group (n=10). Data is presented as the mean \pm standard error. (C) Diversity indexes prior to and following treatment in the long-term survival group (alive ≥ 12 months after treatment). (D) Diversity indexes prior to and following treatment in the short-term survival group (died within 12 months). (E) Changes of TCR β diversity index following vaccine treatment. Blue: Long-term survival group (n=14). Red: Short-term survival group (n=10; P=0.089). TCR β , T-cell receptor β .

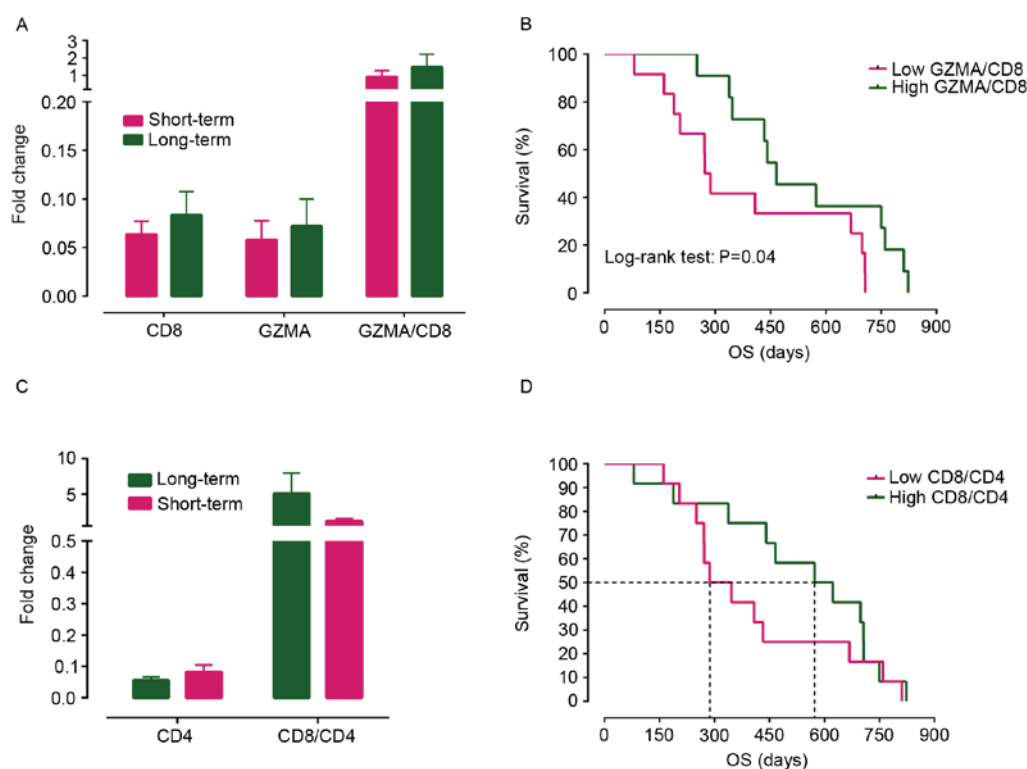


Figure 2. Gene expression analysis. (A) CD8 and GZMA expression normalized to GAPDH. Green: Long-term survival group (n=14). Pink: Short-term survival group (n=10). (B) Kaplan-Meier estimator survival curve separated by the median into high and low GZMA/CD8 ratio prior to treatment (log-rank test, $P=0.04$). (C) CD4 expression levels and CD8/CD4 ratio in the long- and short-term survival groups prior to treatment. Green: Long-term survival group (n=14). Pink: Short-term survival group (n=10). (D) Kaplan-Meier survival curve separated by the median into high and low CD8/CD4 ratio prior to treatment. Median OS time: Low CD8/CD4 group, 316 days; high CD8/CD4 group, 598 days. GZMA, granzyme A; OS, overall survival; CD, cluster of differentiation.

Association between the immunogenic gene expression and survival status of patients with NSCLC treated with cancer peptide vaccines. To assess alterations in T-cell populations in response to treatment, TaqMan assays were performed to analyze the relative expression of CD8, CD4 and GZMA prior to the vaccine treatment. As presented in Fig. 2A, no significant differences were identified for CD8 and GZMA expression levels and the GZMA/CD8 ratio between the long-term and short-term survival groups. However, when the patients were separated into two groups by a median value of the GZMA/CD8 ratio, which may reflect the level of activated cytotoxic T cells, the survival curves were significantly different, favoring the group with a high GZMA/CD8 ratio (log-rank test, $P=0.04$; Fig. 2B). In addition, a tendency was observed for the CD8/CD4 ratio to be increased in the long-term survival group compared with the short-term survival group (this difference was not statistically significant, Fig. 2C). Notably, the median overall survival time in the low CD8/CD4 group was only 316 days compared with 598 days in the high CD8/CD4 group (Fig. 2D).

Discussion

Although numerous attempts have been made to develop an effective peptide cancer vaccine therapy to treat NSCLC, the results have not been positive (9,12,13). The present study aimed to screen possible predictive biomarkers that were able to distinguish responders from non-responders in patients with HLA-A24-positive NSCLC who received a cancer peptide vaccine treatment of a mixture of three peptides. Certain

long-term survivors were observed among these patients who had previously failed standard therapies. Injection site reaction was the most common type of adverse event possibly associated with treatment. One patient in whom a coronary artery stent had been placed for angina succumbed to myocardial infarction as confirmed by autopsy, indicating a non-deniable causal association between treatment and this adverse event.

The immunogenic properties of this vaccine were evaluated through an analysis of the TCR β repertoire in blood samples prior to and following 8 weeks of treatment. The main results included the following: i) A lower DI prior to vaccine treatment appeared to be beneficial, as it was associated with an improved survival status; and ii) a higher ratio of activated cytotoxic T cell at baseline, as indicated by GZMA/CD8 prior to treatment, appeared to be a favorable clinical outcome. A previous study demonstrated that cancer vaccination may cause an increase in circulating tumor antigen-specific T cells (46). However, the profiles of these T cells have not been well characterized with regard to their association with treatment outcomes. The results of the present study demonstrated that patients with a lower baseline DI tended to have a longer survival time. Notably, patients enrolled in the trial had advanced NSCLC refractory to standard chemotherapies. Prior treatment with cytotoxic chemotherapies may lead to generation of cancer-specific antigens that possibly generate immunological effects, although intensive chemotherapy may also kill these CTL clones (47). It was speculated that the residual immunogenic effects of prior chemotherapies led to the polyclonal expansion of T-cell clones and that this

expansion, reflected as a lower DI of the TCR β repertoire, contributed to the positive effect of the vaccine. Therefore, the pretreatment TCR β DI may be used as a predictive marker of the ability of a patient to generate an immune response against either chemotherapeutic or vaccine treatment. Peptide vaccination was reported to boost a preexisting dominant clonotypic response (48). Therefore, a suggestion was made to administer vaccine therapy concomitantly with chemotherapy to generate the most effective immunological effects (49). A trend towards an increased TCR β DI was observed in long-term survivors but not in short-term survivors, supporting earlier evidence that TCR β repertoire diversification due to chemotherapy and vaccination is beneficial in the prevention of immune-resistant mutant cancer cells, since more cancer-specific T-cell clones; particularly cytotoxic T cells are generated to target cancer cells (49). This phenomenon may be explained as a secondary immune response to vaccine treatment. As the immune system (either cytotoxic T cells or macrophages) eliminated greater numbers of cancer cells, phagocytosis of these cells may result in the presentation of cancer-specific antigens by antigen-presenting cells. The results of the present study were consistent with those of a study by Fang *et al* (25), in which patients with NSCLC were demonstrated to benefit from chemotherapy prior to peptide vaccination due to an increase in the TCR repertoire diversity. Therefore, the TCR β DI prior to treatment and the increase in the DI of PBLs following treatment may be used to monitor the responses of patients with NSCLC to the peptide vaccine.

A TaqMan gene expression assay was performed to quantify the relative presence of these two populations in the patient samples in the present study. GZMA has been proposed as a biomarker of activated cytolytic T lymphocytes (50). A high level of GZMA expression indicates a high level of inflammatory cells in allograft, autoimmune diabetes and chronic Chagas' myocardial lesions (51,52). Therefore, GZMA/CD8 ratios were calculated to study the activated subset of cytotoxic CD8 cells. Although no difference was observed in the expression of CD8 or GZMA between the long-term and short-term survivor groups, the results indicated that an increased level of GZMA/CD8 ratio was beneficial for the survival status. Although the difference was not statistically significant, the relative ratio of CD8⁺/CD4⁺ was increased prior to treatment in the long-term survival group. A limitation of the present study was that the limited number of available T lymphocytes did not allow for T-cell sorting prior to sequencing. Therefore, future studies are required to improve the characterization of the specific TCR repertoires of different T-cell populations (CD8⁺, CD4⁺CD25⁺ and CD4⁺CD25⁻), and thus elucidate the specific immunogenic effect of the three-peptide cocktail vaccine.

In conclusion, TCR β DI and the immunogenic markers, including GZMA, may serve as predictive biomarkers for successful cancer vaccine treatment. Limitations of the present study included the limited number of patients and the lack of placebo control arm. Future prospective studies, in which these markers are used to predict the outcomes of vaccine treatment, are warranted.

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