PD-1 expression on peripheral blood T-cell subsets correlates with prognosis in non-small cell lung cancer

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PD-1 expression in peripheral blood T-cells has been reported in several kinds of cancers, including lung cancer. However, the relationship between PD-1 expression in peripheral blood T-cells and prognosis after treatment with a cancer vaccine has not been reported. To elucidate this relationship, we analyzed PD-1 expression in the peripheral blood T-cells of patients with non-small cell lung cancer. The blood samples used in this study were obtained from patients enrolled in phase II clinical trials of a personalized peptide vaccine. Seventy-eight samples obtained before and after a single vaccination cycle (consisting of six or eight doses) were subjected to the analysis. PD-1 was expressed on lymphocytes in the majority of samples. The relative contents of PD1+CD4+ T-cells against total lymphocytes before and after the vaccination cycle correlated with overall survival (OS) with a high degree of statistical significance (P < 0.0001 and P = 0.0014). A decrease in PD-1+CD8+ T-cells after one cycle of vaccination also correlated with longer OS (P = 0.032). The IgG response to the non-vaccinated peptides suggested that the epitope spreading seemed to occur more frequently in high-PD-1+CD4+ T-cell groups. Enrichment of CD45RA+CCR7+ effector-memory phenotype cells in PD-1+ T-cells in PBMCs was also shown. These results suggest that PD-1 expression on the peripheral blood T-cell subsets can become a new prognostic marker in non-small cell lung cancer patients treated with personalized peptide vaccination.

Lung cancer is the most common cancer in the world; annually 1.8 million new cases are diagnosed and 1.6 million people die of the disease.1 Approximately 80% of lung cancers are non-small cell lung cancers (NSCLCs).2 Surgery is the standard treatment in the early stages of NSCLC. However, more than 65% of patients with NSCLC are in advanced stages with locally advanced or metastatic disease.3 Although recent progress with molecular targeted agents, including tyrosine kinase inhibitors of epidermal cell growth factor receptor (EGFR) and anaplastic lymphoma kinase (ALK), as well as progress in the development of antibodies against vascular endothelial cell growth factor (VEGFR), improved the prognosis of NSCLC patients in advanced stages,4,5 new treatment modalities need to be developed. Cancer vaccine therapies are among the promising new therapeutic modalities for NSCLC. We have developed a personalized peptide vaccine (PPV), in which appropriate vaccine peptides are selected from a panel of candidate peptides on the basis of each patient’s HLA-A types and pre-existing anti-cancer immunity.6 Currently, there are 31 CTL-epitope peptide candidates derived from a variety of tumor-associated antigens; these include 12 peptides for HLA-A2 patients, nine peptides for patients with an HLA-A3 super type (A3, A11, A31, or A33), 14 peptides for HLA-A24 patients, and four peptides for HLA-A26 patients.6,7 A maximum of four peptides, which were selected based on patient’s HLA types and pre-existing immunity, were subcutaneously injected with ISA51 VG weekly or bi-weekly. Clinical studies have shown the safety and potential immunological efficacy of these peptides in small cell and non-small cell lung cancers.8,9

Anti-tumor immunity is regulated by several immune checkpoint molecules. Programmed cell death 1 (PD-1) is one of the immune checkpoint molecules expressed on both activated and exhausted T-cells.10 PD-L1, the PD-1 ligand, is expressed on tumor cells and PD-1/PD-L1 interaction provide negative signal for antigen-induced T-cell activation.11 Therefore, T-cell inactivation induced by PD-1/PD-L1 is thought to be a mechanism underlying immunosuppression at the tumor site.11 Several reports have examined PD-1 expression on tumor-infiltrating T-cells, and its correlation with prognosis has been discussed12–19 However, PD-1 expression on the peripheral blood T-cells of cancer patients, particularly in those with lung cancer, has not been sufficiently studied.20–22 In this paper, we analyzed PD-1 expression and other immune checkpoint molecules on peripheral blood T-cells of NSCLC patients and found some correlation with prognosis.
Materials and Methods

Clinical samples. The peripheral blood samples used in this study were obtained from patients enrolled in phase II clinical trials of PPV for advanced NSCLC. The study protocols were approved by the Kurume University Ethics Committee and were registered with the UMIN Clinical Trial Registry, UMIN 1839 and UMIN 2984. The entry criteria and precise vaccination protocols were reported previously. One vaccination cycle consisted of six or eight doses of peptide vaccination. The patient’s blood samples were taken before and after one cycle and stored until use.

Flow cytometric analysis. Peripheral blood mononuclear cells (1 x 10⁷) were suspended in PBS containing 20% human AB serum and incubated for 30 min on ice with appropriate dilution of antibodies. The antibodies used in this study were: anti-CD4-FITC (clone RPA-T4), anti-CD8-PerCP-Cy5.5 (clone RPA-T8), anti-CD279 (PD-1)-APC (clone MIH4), anti-CD152 (CTLA-4)-APC, and anti-CD137 (4-1BB)-APC from BD Biosciences (Franklin Lakes, NJ, USA), anti-CD197 (CCR7)-PerCP-Cy5.5 (clone G043H7), anti-CCR7-Alexa Fluor 488 (clone G043H7), and anti-CD45RA-PE (clone HI100) from BioLegend (San Diego, CA, USA). For the negative controls, APC mouse IgG1(κ) (clone MOPC-21; BD Biosciences) and PE mouse IgG2b (κ) (clone MOPC-11; BioLegend) were used. The stained cells were analyzed on BD FACs Canto II with FACs Diva software (BD Biosciences).

Measurement of IgG and CTL responses. The IgG level in each of the 31 peptide candidates was measured using the Luminex system (Luminex, Austin, TX, USA), as previously reported. The IgG levels were obtained as fluorescence intensity units (FIU). If the level of peptide-specific IgG to at least one of the vaccinated peptides in the post-vaccination plasma was more than twice that in the pre-vaccination plasma, the response was considered augmented. For the analysis of IgG response to the peptides, FIU values were converted to IgG scores, which were calculated by the formula

$$\text{IgG score} = \log_2 (\text{FIU value}/10)$$

The CTL responses were evaluated by IFN-γ ELISpot assay as previously described. Antigen-specific T-cell responses were evaluated by the difference between the number of spots produced in response to each corresponding peptide and that produced in response to the control HIV peptide; a difference of more than 5 spots per 10⁵ PBMCs with statistical significance (P < 0.05) was considered positive or detectable and the subtracted spot numbers are shown. In negative cases, spot numbers are shown as 0. If a post-vaccination value was more than twice the pre-vaccination value with statistical significance (P < 0.05 by t-test), the response was considered augmented. If a pre-vaccination value was 0, more than 30 post-vaccination values were considered augmented responses. The CTL response data will be considered augmented. The CTL response data will be analyzed by the Kaplan–Meier test and the log-rank test for the analysis of the survival curves. Pearson’s χ² test and the ANOVA were used to analyze T-cell responses specific to the vaccinated peptides and IgG responses to the vaccine peptides, respectively. A P-values <0.05 were considered significant. All statistical analyses were performed using JMP version10 software (SAS Institute, Cary, NC, USA).

Results

Patient characteristics. Seventy-eight patients with advanced (stages IIIb and V) or recurrent NSCLC receiving PPV treatment were included in this study. Table 1 shows their characteristics. By the start of PPV treatment, 73 of the 78 patients had failed at least one regimen of chemotherapy: 16, 22, 11, and 24 patients had, respectively, failed 1, 2, 3, or 4–12 regimens. The remaining five patients were naive to any chemotherapy regimens before PPV treatment.

Expression of immune checkpoint molecules on peripheral blood lymphocytes. Expression of two co-inhibitory molecules, CTLA-4 (CD152) and PD-1 (CD279), and one co-stimulatory molecule, 4-1BB (CD137) on lymphocytes of the patients’ PBMCs were examined. Although these three molecules were expressed on PHA-activated lymphocytes, only PD-1 was detectable on non-stimulated lymphocytes of the majority of patients and the other two molecules were rarely expressed on the peripheral blood lymphocytes (data not shown). Therefore, further analyses focused on the expression of PD-1 on patients’ lymphocytes. Figure 1 shows representative staining patterns of PD-1 on CD4+ and CD8+ T-cell subsets as well as total lymphocytes.

PD-1 expression and prognosis. We examined PD-1 expression in T-cells of PBMCs obtained from patients before and after one cycle of six or eight vaccination doses. The median relative contents of PD-1 in CD4+ T-cells against total lymphocytes were 7.35% (range, 2.4–20.1%) and 7.6% (3.4–26.2%) at pre- and post-vaccination; those of PD-1 in CD8+ T-cells against total lymphocytes were 4.4% (1.4–14.3%) and 4.25% (1.6–19.0); and those of PD-1 in lymphocytes against total lymphocytes were 13.85% (6.6–29.2%) and 13.7% (6.0–42.0%).

Subsequently we analyzed the correlation between the relative contents of PD-1 and overall survival (OS) by a Kaplan–Meier plot (Fig. 2). “Median” of % PD-1 cells were used for the definition of “high” and “low” contents of PD-1 cells. The relative contents of PD1+CD4+ T-cells against total lymphocytes of pre-vaccination samples correlated to OS with
The pre- and post-vaccination contents of PD-1+CD4+ T-cells were significantly correlated ($P < 0.0001$). In contrast, there was no correlation between the pre-vaccination contents of PD-1+CD4+ T-cells and the difference of PD1+CD8+ T-cells between pre- and post-vaccination, or between the post-vaccination contents of PD-1+CD4+ T-cells and the difference in PD1+CD8+ T-cells (data not shown). Univariate analysis indicated that the high-contents of pre- and post-vaccination PD-1+CD4+ T-cells were correlated to longer OS, as was the decrease in PD1+CD8+ T-cells. Multivariate analysis showed that the post-vaccination contents of PD-1+CD4+ T-cells and the decrease in PD1+CD8+ T-cells were independent factors for longer OS, and the combination of these two factors is a stronger prognostic factor than either factor alone (Table S1).

Neither the high- and low-content nor the decreased and non-decreased groups differed significantly in any of the patient’s background factors, such as age, gender, stages, and histology, except for OS (data not shown).

**PD-1 expression and CTL response.** We analyzed the relationships between CTL responses to the vaccinated peptides and the PD-1+CD4+ T-cell contents at pre- and post-vaccination, as well as the relationships between the CTL responses and the difference in PD1+CD8+ T-cell contents. Forty-nine patients were included in this analysis, since no sufficient blood samples from pre- and post-vaccination were available from the remaining patients. A response was considered augmented if the IFN-γ ELISPOT-cell numbers more than doubled after the vaccination cycle against at least one vaccinated peptide. The percentages of CTL-response-augmented cases among the 49 cases in the PD-1+CD4+ T-cell high- and low-content groups were 47.6% (10/21) and 47.0% (13/28), respectively, at pre-vaccination; 47.4% (9/19) and 46.7% (14/30) at post-vaccination; and 50.0% (12/24) and 44.0% (11/25) in the decreased and non-decreased groups of PD-1+CD8+ T-cell contents after the cycle (Fig. 3). These groups showed no differences in CTL responses for any parameter.

**PD-1 expression and IgG response.** We analyzed the relationships between IgG responses to the vaccinated peptides and the PD-1+CD4+ T-cell contents at pre- and post-vaccination, as well as the relationship between the IgG responses and the difference in PD1+CD8+ T-cell contents. For each parameter, changes in the sums of IgG scores after the cycle were compared between the groups. As shown in Figure 4(a), IgG responses to the vaccinated peptides in the PD-1+CD4+ high-content group were significantly higher than those in the PD-1+CD4+ low-content group both before and after vaccination ($P = 0.0267$ and $P = 0.0308$, respectively). Analysis of the IgG subclass in the representative cases indicated that IgG1, IgG2, and IgG3 were equally dominant in both the pre- and post-vaccination samples from the PD-1+ T-cell high- and low-content groups (data not shown).

We also measured IgG responses to the peptides that were not used for the vaccination, designated as non-vaccinated peptides, to examine how vaccination spread the epitope, and we analyzed the correlation between the groups for each parameter. An IgG response was considered augmented if the IgG value against the peptide was more than doubled after the cycle. The number of peptides corresponding to the augmented IgG responses was compared between the two groups for each parameter. As shown in Figure 4(b), there were significantly more peptides in the PD-1+CD4+ high-content group than in the PD-1+CD4+ low-content group at post-vaccination ($P = 0.0079$). Changes in the sums of IgG scores against the nonvaccinated peptides after the cycle were also compared.
between the groups for each parameter (Fig. 4c). The changes in the PD-1+CD4+ high-content group were also significantly higher than those in the PD-1+CD4+ low-content group at post-vaccination ($P = 0.0016$).

**Effector memory T-cells are enriched in PD-1+ T-cells.** We further analyzed the cell surface expression of CD45RA and CCR7 on PD-1+ T-cell subsets of NSCLC patients ($n = 18$). Representative results are shown in Figure 5. The majority of PD-1+CD4+ T-cells expressed the CD45RA+CCR7+ effector-memory phenotype (median = 75.6%, range 57.2–91.3%), and the median frequencies of CD45RA+CCR7+ central memory and CD45RA–CCR7–naive phenotype cells were 10.4% (5.4–21.6%) and 7.55% (0.2–19.5%), respectively. In contrast, the median frequency of CD45RA+CCR7+ effector-memory and CD45RA–CCR7–naive phenotype cells were respectively 37.7% (19.1–78.4%) and 39.95% (4.5–61.7%) in PD-1+CD8+ T-cells. Similarly, in CD8+ T-cells the effector-memory phenotype of cells was dominant (median = 83.85%, 47.9–92.6%) in PD-1+ cells. Similar results were also obtained from PBMCs of healthy donors (data not shown). The enrichment of effector-memory T-cells in PD-1+ T-cells in PBMCs was consistent with previous reports of healthy donors and HCV/HIV co-infected patients. (23,24)

**Discussion**

For the development of new treatment modalities, the co-development of new biomarkers is important. Particularly for cancer vaccines, it is crucial to identify appropriate biomarkers to predict overall survival, since only some patients will show clinical benefit. Our previous clinical studies of PPV indicated that several biomarkers were significantly correlated with the OS. (8,9,25,26) In the case of NSCLC, a high CRP level at pre-vaccination and a low frequency of CD26+ T-cells in the peripheral blood after one vaccination cycle were significant biomarkers of unfavorable OS. (9) However, most of these biomarkers are prognostic but not predictive. The MST of a phase II study of PPV in 44 patients with refractory NSCLC,
including 4 stage IIIb, 22 stage IV, and 15 recurrent, was
304 days, with a 1-year survival rate of 42%. If appropriate predictive biomarkers are
identified, the clinical efficacy of PPV might be improved.

We have demonstrated in this study that relatively high contents of PD-1⁺CD4⁺ T-cells against total lymphocytes of blood samples obtained before and after a vaccination cycle were correlated with longer OS. Among them, high contents of post-vaccination PD-1⁺CD4⁺ T-cells and a decrease in PD-1⁺CD8⁺ T-cells were correlated to high contents of CD26⁺ T-cells, in contrast, no significant correlation was observed between the CRP levels and the contents of PD-1⁺ T-cell sub-
sets (data not shown). A similar correlation, between a high frequency of PD-1⁺CD4⁺ T-cells in pre-treatment blood samples and prolonged OS, was reported in a clinical study of prostate GVAX plus ipilimumab (anti-CTLA-4) in patients with metastatic castration-resistant prostate cancer. Expression of PD-1 on peripheral blood T-cells of patients with malignant tumor has been reported. If appropriate predictive biomarkers are identified, the clinical efficacy of PPV might be improved.

Most of the previous reports suggested that PD-1 is a marker of exhausted T-cells, and some of these reports described the dysfunction of tumor-infiltrating PD-1⁺CD8⁺ T-cells. However, the functional difference between PD-1⁺ and PD-1⁻ T-cells in the peripheral blood has not been clarified satisfactorily. Follicular helper T-cells (Tfh) have recently been identified and those surface phenotype is CXCR5⁺PD-1⁺. Only a small part of PD-1⁺CD4⁺ cells found in this study expressed CXCR5, suggesting these cells are different from Tfh (Fig. S1).

PD-1 and CD45RA CCR7 on PD-1 T-cell subsets of non-small cell lung cancer (NSCLC) patients were analyzed. Representative data are shown.

P-values were calculated by ANOVA.

Fig. 4. (a) The correlation between the relative contents of PD-1⁺ T-cell subsets and the IgG response to the peptides used for vaccination was analyzed. Changes in the sums of IgG scores after one cycle of vaccination were compared between the two groups for each parameter. (b) The number of non-vaccinated peptides with enhanced IgG responses after one cycle of vaccination was compared between the two groups for each parameter. (c) Changes in sums of scores of IgG against non-vaccinated peptides after one cycle of vaccination were compared between two groups for each parameter. P-values were calculated by ANOVA.

Fig. 5. Cell surface expression of CD45RA and CCR7 on PD-1 T-cell subsets of non-small cell lung cancer (NSCLC) patients were analyzed. Representative data are shown.
healthy adults humans are effector memory cells rather than exhausted cells. In the present study, we analyzed the phenotypes of PD-1+ T-cells in the peripheral blood and found that the majority of PD-1+CD4+ and PD-1+CD8+ T-cells expressed the CD45RA CCR7+ effector-memory phenotype. Effector-memory T-cells are home to peripheral tissues and can rapidly produce effector cytokines such as IFN-γ upon antigen stimulation. Therefore, PD-1+ T-cells observed in the peripheral blood of patients with NSCLC may not be exhausted or tolerant T-cells. Our preliminary study using anti-PD-L1 blocking antibody also supported this possibility. Namely, anti-PD-L1 antibody augmented the anti-CD3/CD28-induced IFN-γ response of PD-1+ tumor-infiltrated T-cells (TILs) of NSCLC patients, suggesting that the activation of PD-1+ TILs is suppressed by PD-1/PD-L1 pathway. In contrast, the addition of anti-PD-L1 was less effective in such a stimulation induced IFN-γ response of PD-1+ peripheral blood T-cells from the same donor, although PD-L1 was similarly expressed in both TILs and PBMCs (Fig. S2). Another explanation of the correlation between an increased frequency of PD-1+CD4+ T-cells in the peripheral blood and better prognosis is the different distribution of PD-L1 in the peripheral lymph nodes and cancer tissues. It is well known that PD-L1 expression is upregulated in most cancer tissues. It is unknown whether PD-1 on CD4 helper T-cells supplies a negative signal at lymph nodes in which PD-L1 is not upregulated, and PD-1+ regulatory T-cells may suppress its function at tumor sites where PD-L1 is upregulated. If PD-1+ T-cells function as effector memory T-cells, it is hard to explain why the decrease in PD-1+CD8+ T-cells after vaccination correlates to longer OS. One possibility is the vaccination regimen used in this study induced the efficient recruitment of PD-1+CD8+ T-cells into the tumor region, resulting in a decrease in the cell population in peripheral blood. Indeed, increased infiltration of CD8+ T-cells into the tumor region had been confirmed in vaccinated patients with prostate cancer. Further precise studies are needed to clarify these issues. We showed that IgG response correlated to high content of PD-1+CD4+ T-cells, which in turn correlated to good prognosis, and that CTL response did not correlate to any PD-1+ T-cell parameters. However, CTL response rather than IgG response was the good marker of OS (T. Yamada et al., in preparation). Dissociation between the CTL-response and PD-1+CD8+ T-cell contents might be due to the low contents of PD-1+ cells in CD8+ T-cells, in which both the PD-1+ and PD-1- cells have a potential of CTL. The present study included patients treated with vaccine monotherapy or a combination of vaccination with chemotherapy or molecular targeting therapy; these therapies might affect the CTL response as well as PD-1 expression and prognosis. To clarify these issues, further study using segment analysis with large numbers of patients is needed.

Many clinical trials of anti-immune checkpoint antibodies and antagonists are now under way. In the case of PD-1, clinical studies of several anti-PD-1 antibodies were conducted in patients with various malignant tumors including NSCLC. All of those studies found that these anti-PD-1 antibodies were tolerable and showed promising clinical benefits. The combination of cancer vaccines and immune checkpoint blockades will become a new trend in cancer immunotherapy. PD-1 as well as other checkpoint molecules on peripheral blood T-cells become target molecules in combination therapy. Thus the expression of PD-1 on T-cells may provide different predictions in combination therapy.

In conclusion, we found that the relatively high contents of PD-1+CD4+ T-cells in the peripheral blood, as well as the decrease in PD-1+CD8+ T-cells after vaccination, were correlated with longer OS. These results suggest that PD-1 expression in peripheral blood T-cell subsets will become a new prognostic marker for NSCLC patients treated with personalized peptide vaccination.

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Disclosure Statement

Akira Yamada is a board member of the Green Peptide Co. (Kurume, Japan).

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