Association between soluble immune mediators and tumor responses in patients with non-small cell lung cancer treated with anti-PD-1 inhibitor

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The changes in the plasma levels of CXCL2 and MMP2 after anti-PD-1 treatment were associated with PFS, and these changes were maintained during the course of anti-PD-1 therapy in the patients with better clinical outcomes. Since CXCL2 and MMP2 can be easily measured by minimally invasive blood sampling, they could be useful for monitoring of clinical outcomes in NSCLC patients receiving PD-1 inhibitor therapy.

Abstract

Although programmed death (PD)-1 immune checkpoint therapies target the immune system, the relationship between inflammatory factors and the clinical outcome of anti-PD-1 therapy for non-small cell lung cancer (NSCLC) is not fully understood. Here we examined the association between soluble immune mediators and the outcome of treatment with PD-1 inhibitors in patients with advanced/recurrent NSCLC. In two independent cohorts, we assessed the levels of 88 different soluble immune mediators in peripheral blood before and after anti-PD-1 treatment, and evaluated their associations with clinical outcomes. In the training cohort, the plasma levels of chitinase 3-like-1 and GM-CSF before treatment (P = 0.006 and P = 0.005, respectively) and changes in the plasma levels of CXCL2, VEGF, IFNα2, and MMP2 after treatment (P <0.001, P = 0.019, P = 0.019, and P = 0.012, respectively) were significantly correlated with PFS. The change in the plasma CXCL2 level was also significantly associated with treatment-related AEs (P = 0.017). In the validation cohort, however, only the changes in the plasma levels of CXCL2 and MMP2 after treatment were associated with PFS (P = 0.003 and P = 0.006, respectively), and these changes were maintained during the course of anti-PD-1 therapy in patients who showed better clinical outcomes, even in those with tumor pseudoprogression. Since CXCL2 and MMP2 can be easily measured by minimally invasive blood sampling, they could be useful for monitoring of clinical outcomes in NSCLC patients receiving PD-1 inhibitor therapy.

Introduction

Lung cancer is a leading cause of cancer-related death worldwide[1]. The programmed death (PD)-1 receptor expressed on immune cells, including T cells, B cells, NK cells and macrophages, is activated by binding to two ligands, PD-L1 and PD-L2, thus suppressing immune cell activation and promoting the ability of tumor cells to escape immune surveillance[2]. PD-1/PD-L1 inhibitors that restore the immune system's capacity to recognize and eradicate tumors have emerged as a promising treatment option for advanced cancer[3-9]. For example, PD-1 inhibitors such as nivolumab and pembrolizumab have recently been approved for treatment of advanced non-small cell lung cancer (NSCLC), resulting in their widespread clinical use.

Since PD-1 immune checkpoint therapies target the immune system, their characteristics in terms of immune-related adverse events (AEs) and clinical outcomes seem to differ considerably from those of traditional therapies[3-9]. For example, PD-L1 expression on tumor cells or immune cells, as assessed by immunohistochemistry (IHC), is reportedly associated with higher response rates in NSCLC patients receiving anti-PD-1 therapy[5,10,11]. In addition, recent reports have suggested that immune-related AEs may be associated with clinical responses to anti-PD-1 therapy[10-12]. Given that immune-mediated inflammatory responses might play an important role in the antitumor effects and/or treatment-related AEs of anti-PD-1 therapy, it may be important to examine and validate the inflammatory factors related to them. In the present study, therefore, using peripheral blood samples, we investigated whether soluble immune mediators were associated with clinical outcomes in patients with NSCLC treated with anti-PD-1 inhibitor.

Materials and Methods

Overview of study procedure

In this study, we adopted a two-stage approach involving a training stage and a validation stage. The purpose of the training cohort was to screen soluble immune mediators related to clinical outcomes. To this end, we analyzed the associations between 88 soluble immune mediators, including cytokines, chemokines, and growth factors, and clinical outcomes such as progression-free survival (PFS) and occurrence of adverse events (AE). As the training cohort included a small number of patients and many candidate immune mediators were examined, there was some concern that a high degree of false positivity might have affected our conclusions. Therefore, the second stage (involving the validation cohort) was used for validating our initial findings, i.e. for examining associations between the soluble immune mediators identified in the first stage and clinical outcomes.

Patients

The training cohort comprised 27 patients with advanced/recurrent NSCLC receiving the PD-1 inhibitor, nivolumab, at Kurume University Hospital (Kurume, Japan). Peripheral blood samples were taken from these patients before (n = 27) and after (at 6 weeks from the date of the first dose; n=20) anti-PD-1 therapy. The validation cohort comprised 50 patients with advanced/recurrent NSCLC receiving nivolumab or pembrolizumab as the PD-1 inhibitor at Kurume University Hospital (n = 34) or Kanagawa Cancer Center (Yokohama, Japan; n = 16). Peripheral blood samples were taken before (n = 50) and after (at 6 weeks

from the date of the first dose; n = 43) anti-PD-1 therapy. In both cohorts, sampling of peripheral blood was repeated every 6-12 weeks in the patients who continued anti-PD-1 therapy (Figure 1A). All eligible patients received nivolumab (3 mg/kg/body) every 2 weeks or pembrolizumab (200 mg/body) every 3 weeks. The present study was conducted in accordance with the provisions of the Declaration of Helsinki, and was approved by the Institutional Review Boards of Kurume University Hospital and Kanagawa Cancer Center. Informed consent was obtained from all of the patients involved in the study after its nature and possible consequences had been explained.

Immunohistochemistry (IHC) analysis

Paraffin-embedded tissue samples were cut into sections 4 μm thick and placed on coated glass slides. Primary antibodies (with dilutions) directed against the following markers were employed: PD-L1 (1:100, clone E1L3N; Cell Signaling Technology, Inc., Danvers, MA), CD8 (1:200, clone 4B11, Leica Microsystems, Newcastle-upon-Tyne, UK), CXCL2 (1:300, PeproTech, NJ, USA), and MMP2 (1:100, clone D4M2N, Cell Signaling Technology, Inc.). Briefly, slides were heat-treated using epitope retrieval solution 2 (pH 9.0) for 30 min, and then incubated with the antibodies using a Bond-III autostainer (Leica Microsystems). This automated system used a refined polymer detection system with horseradish peroxidase (HRP)-polymer as the secondary antibody. The slides were visualized using 3,3' diaminobenzidine (DAB) as the chromogen.

Measurement of soluble immune mediators in plasma

To comprehensively detect the levels of soluble immune mediators (cytokines, chemokines, growth factors, etc.) in plasma before and after anti-PD-1 treatment, a bead-based multiplex assay was used. For this assay, soluble immune mediators were measured in 100-µl aliquots of two-fold-diluted plasma using a Bio-Plex 200 system (Bio-Rad Laboratories, Hercules, CA) in accordance with the manufacturer's instructions. Analyte kits from Bio-Rad Laboratories were used for measurement of the following 88 different soluble immune mediators: IL-1Rα, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12 (p40), IL-12 (p70), IL-13, IL-15, IL-16, IL-17A, IL-19, IL-20, IL-22, IL-26, IL-27, IL-28A, IL-29, IL-32, IL-34, IL-35, IFN-α2, IFN-β, IFN-γ, TNF-α, GM-CSF, 6Ckine, BCA-1, CTACK, ENA-78, eotaxin, eotaxin-2, eotaxin-3, fractalkine, GCP-2, Gro-α, CXCL2 (Gro-β), IP-10, I-TAC, MIP-1α, MIP-1δ, MIP-3α, MIP-3β, MPIF-1, SCYB16, SDF-1 α+β, TARC, TECK, I-309, MCP-1, MCP-2, MCP-3, MCP-4, MDC, MIF, MIG, VEGF, APRIL, BAFF, sCD30, sCD163, chitinase 3-like-1, gp130, IL-6Rα, LIGHT, MMP-1, MMP-2, MMP-3, osteocalcin, osteopontin, pentraxin-3, sTNF-R1, sTNF-R2, TSLP, TWEAK, VEGF, IgG1, IgG2, IgG3, IgG4, IgM, and IgA.

Statistical analysis

The severity of AEs was graded using the National Cancer Institute Common Terminology Criteria for Adverse Events version 4.0, and their causal association with anti-PD-1 treatment was determined by the investigators. Best overall response was defined as the best response designation, recorded between the date of the first dose of PD-1 inhibitor and the date of initial objectively documented tumor progression as determined by

the Response Evaluation Criteria in Solid Tumors (RECIST) 1.1 criteria[13]. Pseudoprogression was defined as RECIST progressive disease followed by a delayed clinical response at later assessment. Progression-free survival (PFS) was defined as the period from the date of the first dose to the date of disease progression or death due to any cause. Overall survival (OS) was defined as the period from the date of the first dose to the date of death from any cause.

To examine the association between soluble immune mediators and clinical outcomes, we conducted two types of analysis: one examined the association between baseline measurements and clinical outcomes (baseline analysis), and the other examined the association between changes in soluble immune mediator levels from the baseline up to 6 weeks and clinical outcomes (change analysis). In the change analysis, a 6-week landmark survival analysis was performed by including only patients alive at follow-up starting at 6 weeks after the first treatment in both the training (n = 20) and validation (n = 43) cohorts.

In the training cohort, we enrolled only 27 patients for the baseline analyses and 20 patients for the change analyses. This precluded the use of complicated statistical models. Accordingly, we employed regression analysis only for each soluble immune mediator as a single explanatory variable (univariate analysis). The Cox regression model was employed for PFS, and the logistic regression model was employed for AEs. To avoid loss of power, we included each soluble immune mediator in regression analysis as a continuous variable. Since the purpose of the training cohort was to screen soluble immune mediators related to clinical outcomes, we were not overly concerned about false positivity. Therefore, we did not control for multiplicity in testing, and employed P <0.02 as a screening tool for

identification.

The same approach was also employed in the validation cohort with a two-sided significance level of $P<0.05$. Strictly speaking, the issue of multiplicity may have arisen because several soluble immune mediators and outcomes were analyzed in the validation cohort. However, multiplicity adjustment was not applied because the validation cohort was still exploratory in nature. To demonstrate significance of the soluble immune mediators finally identified, Kaplan-Meier plots stratified according to their changes after treatment were used. The Pearson product-moment correlation coefficient was used to determine the correlations between the changes in the levels of selected soluble factors and the changes in sum of longest tumor diameter. All of the statistical analyses were conducted using the R-language for statistical computing (version 2.9.0), JMP version 11 (SAS Institute Inc., Cary, NC) or Graph Pad Prism version 6.07 for Windows (GraphPad Software, La Jolla, CA; www.graphpad.com).

Results

Patient characteristics

The training cohort included 27 patients with advanced/recurrent NSCLC who were started on anti-PD-1 therapy between February 2016 and September 2016 (Table 1). The median age of the patients overall was 69 years (range, 53 to 82 years); 20 (74%) patients were male; 17 (63%) had a good PS [Eastern Cooperative Oncology Group (ECOG) 0 or 1]; 18 (67%) and 7 (26%) had adenocarcinoma and squamous cell carcinoma, respectively; 7 (26%) and 1 (4%) had EGFR and ALK mutation, respectively; 7 (26%) had never smoked; 22 (81%) had stage IV or recurrent cancer. Baseline PD-L1 expression was weakly positive (1-49% of tumor cells) and strongly positive (>50% of tumor cells) in 4 (21%) and 4 (21%), respectively, of the 19 patients whose tissue samples were available. All of the patients received nivolumab after completing at least one chemotherapy regimen previously. The median follow-up period was 198 days (range, 64 to 330 days). Treatment-related AEs of any grade occurred in 44% of the patients, as listed in Table S1.

The validation cohort included 50 patients with advanced/recurrent NSCLC who were started on anti-PD-1 therapy between October 2016 and September 2017 (Table 1). The median age of the patients overall was 67 years (range, 37 to 84 years); 36 (72%) patients were male; 39 (78%) had a good PS (ECOG 0 or 1); 34 (68%) and 7 (14%) had adenocarcinoma and squamous cell carcinoma, respectively; 8 (16%) and 1 (2%) had EGFR and ALK mutation, respectively; 11 (22%) had never smoked; 39 (78%) had stage IV or recurrent cancer. Baseline PD-L1 expression was weakly positive and strongly positive in 13 (28%) and 27 (57%), respectively, of the 47 patients whose tissue samples

were available. Sixteen (32%) patients had previously untreated advanced/recurrent NSCLC and received pembrolizumab as the first line therapy. Of the remaining 34 patients, 24 (48%) and 10 (20%) received nivolumab and pembrolizumab as second or later line therapy, respectively. The median follow-up period was 158 days (range, 16 to 449 days). Treatment-related AEs of any grade occurred in 48% of the patients, as listed in Table S1.

Association between clinicopathological characteristics and PFS or treatment-related AEs

We first searched for pretreatment clinicopathological characteristics that might be correlated with PFS or treatment-related AEs in the training and validation cohorts (Table S2). A better PS before treatment was associated with improved PFS in both cohorts (P = 0.003 and P =0.018, respectively), whereas other factors such as age, gender, histology, smoking status, disease stage, and the number of prior systemic treatments had no association with PFS or AEs. In addition, neither the neutrophil-lymphocyte ratio, driver mutation, nor PD-L1 expression was significantly associated with PFS or AEs.

Identification of soluble immune mediators associated with the efficacy and safety of anti-PD-1 therapy

To reveal which soluble immune mediators were associated with PFS or treatment-related AEs, we next examined the levels of 88 different soluble immune mediators in pretreatment plasma samples from the training cohort (Table 2). Cox regression showed that the levels of chitinase 3-like-1 and GM-CSF were significantly

correlated with PFS (P = 0.006 and P = 0.005, respectively). We also analyzed whether changes in the plasma levels of 88 different soluble immune mediators were associated with PFS or treatment-related AEs in the training cohort. Cox regression showed that changes in the levels of CXCL2, VEGF, IFNα2, and MMP2 were significantly correlated with PFS (P <0.001, P = 0.019, P = 0.019, and P = 0.012, respectively). In addition, the change in the level of CXCL2 was also significantly associated with treatment-related AEs (P = 0.017).

Validation of the selected soluble immune mediators

The roles of the six soluble immune mediators selected in the training cohort were tested in the validation cohort (Table 2). Lower levels of chitinase 3-like-1 in pretreatment plasma tended to be related to better PFS, although not to a statistically significant degree (P = 0.068). The level of GM-CSF in pretreatment plasma was below the minimum detectable limit in all patients in the validation cohort, possibly due to measurement issues. In contrast, changes in the levels of CXCL2 and MMP2 were significantly correlated with PFS (P = 0.003 and P = 0.006, respectively); decreasing CXCL2 levels and increasing MMP2 levels reflected a better response to anti-PD-1 therapy. A decreasing level of CXCL2 also tended to be related to a higher prevalence of treatment-related AEs, but not to a statistically significant degree (P = 0.108).

Association between changes in plasma CXCL2 and MMP2 levels and clinical outcomes after PD-1 inhibitor treatment

Based on the results of Cox regression in the training and validation cohorts, we stratified the patients into two groups according to the change in the plasma levels of CXCL2 and MMP2. Seven (35%) of 20 training cohort patients and 15 (35%) of 43 validation cohort patients showed decreasing levels of plasma CXCL2. Figure 1B shows the pre- and post-treatment levels of CXCL2 in the patients grouped according to objective tumor response. The changes in CXCL2 levels were significantly associated with clinical responses in both the training and validation cohorts (P=0.001 and P <0.001, respectively). A decrease in the plasma level of CXCL2 was also significantly associated with longer PFS in both the training and validation cohorts (P = 0.008 and P = 0.033, respectively) (Figure 1C). A decreasing level of CXCL2 tended to be related to longer OS in the training and validation cohorts, but not to a statistically significant degree (P = 0.089 and P = 0.082, respectively) (Figure 1C). Regarding the association between changes in plasma CXCL2 levels and clinical outcomes after PD-1 inhibitor treatment, the validation cohort showed a trend similar to that in the training cohort.

Sixteen (70%) of 20 training cohort patients and 22 (51%) of 43 validation cohort patients showed increasing levels of plasma MMP2. The changes in MMP2 levels were significantly associated with clinical responses in the training cohort but not in the validation cohort (P=0.044 and P = 0.061, respectively) (Figure 1B). An increasing level of MMP2 was significantly associated with longer PFS (P = 0.035) in the validation cohort and tended to be related to longer OS (P = 0.074 and P = 0.092, respectively) in both cohorts (Figure 1D).

We also examined whether PD-L1 expression on tumor cells was associated with

changes in the plasma level of CXCL2 or MMP2. However, there was no statistically significant relationship between PD-L1 expression and changes in the plasma level of CXCL2 or MMP2 (Table S3). Of note, the changes in the plasma CXCL2 levels, but not MMP2, after treatment were positively correlated with changes in the sum of the longest diameter of target lesions (r = 0.470, P < 0.001; r = -0.400, P = 0.001; respectively) in 63 patients whose pre- and post-treatment plasma samples were available among both the training and validation cohorts (Supplementary figure S1).

Longitudinal analysis of the plasma levels of soluble immune mediators

To further address how dynamic changes in the plasma levels of CXCL2 and MMP2 might affect clinical outcomes, we analyzed the time courses of their titers in the patients by sequential sampling, as shown in Figure 2A. Of 63 patients whose pre- and post-treatment plasma samples were available among both cohorts, 31 patients (49%) stopped the treatment within one month of the last sample collection due to disease progression. Of the 31 patients discontinuing the treatment, 24 (77%) exhibited a continuous increase of CXCL2 above the pretreatment baseline level until disease progression, and 16 (52%) patients showed decreasing levels of MMP below the pretreatment baseline level before disease progression. On the other hand, 32 (51%) patients had still been receiving anti-PD-1 treatment at the time of analysis. Of these 32 patients, 22 (69%) showed a sustained reduction of CXCL2 below the pretreatment baseline level, and 17 (53%) exhibited sustained elevation of MMP2 above the baseline.

Figure 2B shows representative examples (P3 and P25) of patients who received

nivolumab and demonstrated remarkable anti-tumor responses. Both of them had shown below-baseline levels of CXCL2 and above-baseline levels of MMP2 during tumor shrinkage. One of them (P25) showed pseudoprogression (34% increase), followed by a significant reduction (79%) of tumor volume.

Expression of CXCL2 and MMP2 after treatment with pembrolizumab

The clinical course of patient P49 is shown in Figure 3A. An 83-year-old Japanese woman who had no smoking history was diagnosed as having stage IV (cT4N2M1a) lung adenocarcinoma with ALK fusion. Although the patient was successfully treated with alectinib initially, her tumors relapsed after 6 months. As re-biopsy of the tumor revealed that it had 80% PD-L1 expression, the patient was administrated pembrolizumab. After one cycle of pembrolizumab, the patient initially showed an increase in the size of the tumor lesions, and new lesions appeared in the right lung, However, during the 2 months thereafter, gradual tumor regression was observed. Although some tumor lesions continued to show shrinkage, the primary tumor showed gradual progression. Despite re-administration of pembrolizumab, the patient eventually died of brain infarction. Because the bereaved family provided informed consent, autopsy was performed. The plasma levels of CXCL2 and MMP2 before and after the treatment with pembrolizumab are shown in Figure 3B. The plasma level of CXCL2 was decreased, whereas that of MMP2 was increased after pembrolizumab treatment. We also evaluated the expression of CD8, CXCL2 and MMP2 in tissue samples before and after pembrolizumab. Immunohistochemistry analysis of the tumor tissue revealed that CXCL2 was highly

expressed on macrophages and neutrophils before anti-PD-1 treatment, but was substantially reduced after the treatment, whereas tumor expression of MMP2 was shown to increase after the treatment (Figure 3C).

Discussion

Although PD-1 immune checkpoint inhibitors have recently emerged as a new therapeutic strategy for advanced NSCLC, there has been a paucity of relevant clinical markers that can be used for monitoring of clinical responses to them[14]. Antitumor immunity is regulated by immune checkpoint molecules on immune cells such as T cells, B cells, NK cells and macrophages, as well as through multiple interactions among different types of cells and factors such as cytokines, chemokines, and soluble receptors, making up the tumor microenvironment[15]. In the present study, we investigated associations between soluble immune mediators and clinical outcomes in patients with NSCLC treated with anti-PD-1 inhibitor in two different cohorts. We found that decreasing plasma CXCL2 levels and increasing plasma MMP2 levels after treatment were significantly associated with improved PFS, and that most of the long responders to anti-PD-1 therapy maintained their decrease in CXCL2 and increase in MMP2 during the course of treatment. These results suggest that changes in the levels of CXCL2 and MMP2 might become a useful parameter for monitoring the tumor immune response and effect of anti-PD-1 therapy.

The tumor microenvironment consists of tumor-infiltrating myeloid cells, such as tumor-associated macrophages and neutrophils and myeloid-derived suppressor cells (MDSCs), which are critical contributors to suppression of the immune response[15]. Targeting of tumor-associated myeloid cells for elimination or conversion to their immunostimulatory state has thus emerged as a novel strategy. CXCL2 has been reported to promote recruitment of MDSCs to the tumor bed via signaling mediated by CXCR2 (the receptor for CXCL2)[16]. Interestingly, a recent study using mouse models has demonstrated

that the anti-tumor effect of anti-PD-1 was significantly enhanced when tumor trafficking of MDSCs was inhibited by CXCR2 deficiency or treatment with anti-CXCR2 monoclonal antibody[17]. These results suggest that the post-treatment decrease in the level of CXCL2 might prevent CXCR2-mediated MDSC trafficking toward tumors, leading to enhanced efficacy of PD-1 checkpoint blockade. CXCL2 is known to be produced mainly by myeloid cells, including macrophages and neutrophils[18,19]. Therefore, as shown in Figure 3, the changes in plasma CXCL2 levels might reflect CXCL2 production by tumor-associated macrophages and neutrophils in the tumor microenvironment. Since anti-PD-1 therapy has been shown to exert a direct effect on tumor-associated M2 macrophages with high levels of PD-1 expression[20], it is possible that direct PD-1 blockade on macrophages might inhibit CXCL2 production. Alternatively, the decreased CXCL2 levels might simply be the result of decreased numbers of tumor-associated macrophages and neutrophils after anti-PD-1 treatment, as the changes in the plasma CXCL2 levels tended to be positively correlated with changes in the sum of the longest diameter of target lesions (Fig S1; $r = 0.470$, $P < 0.001$). Further studies to explore the precise mechanisms responsible for changes in plasma CXCL2 levels after anti-PD-1 therapy would be warranted.

Immune-mediated anticancer mechanisms are dependent on close contact between immune cells and cancer cells. However, previous reports have shown that many extravasated lymphocytes remain localized in the stroma surrounding the tumor[21,22], thus limiting the efficiency of cancer immunotherapy[23]. The present study demonstrated that an increase in the plasma level of MMP2 after treatment was significantly associated with the efficacy of anti-PD-1 therapy. Since MMP2 has been shown to have a key role in

degradation of the extracellular matrix[24], it is possible that an increase in MMP2 after anti-PD-1 therapy might accelerate lymphocyte migration to tumor nests. MMP2 is known to be produced by stromal or tumor cells in the tumor microenvironment, but the changes in the plasma MMP2 level were not positive correlation with those in tumor burden (Fig S1). Since there have been no reports on the relationship between MMP2 and anti-PD-1 treatment, the mechanisms by which plasma MMP2 levels were increased after anti-PD-1 treatment remain unknown. Interestingly, the level of MMP2 in peripheral blood is reported to be elevated in patients with autoimmune disease[25,26]. In this study, the incidence of treatment-related AEs tended to increase in PD-1 inhibitor-treated patients with increasing levels of MMP2 in the training and validation cohort, although not to a significant degree ($P = 0.053$ and $P = 0.067$, respectively). The increased level of MMP2 might thus be related to autoimmune responses to PD-1 inhibitors. To clarify the production mechanism and role of MMP2 after anti-PD-1 treatment, further investigations are needed.

Pseudoprogression, which has been defined as a transient increase in the size of existing tumor lesions evident on imaging, or the appearance of new lesions in patients undergoing immunotherapy for cancer[27], has sometimes been mistaken for true progression in patients receiving anti-PD-1 therapy[28]. In this study, three (4%) of 77 patients showed pseudoprogression; all three showed continuous reduction of CXCL2 below the pretreatment baseline level, and two (66%) exhibited continuous elevation of MMP2 above the baseline. These results suggest that monitoring the plasma levels of CXCL2 and MMP2 might contribute to distinguishing between pseudo- and true progression.

Immune-related AEs are reportedly associated with clinical responses to immune

checkpoint inhibitors[10-12,29,30]. Similarly, the present study also showed that patients who suffered treatment-related AEs had a significantly longer PFS and OS (Supplementary figure S2) in both cohorts. These results suggest a close relationship between immune-mediated inflammatory disorders such as autoimmune diseases and the therapeutic effects of anti-PD-1 therapy. However, a significant correlation between changes in the plasma CXCL2 level and the prevalence of treatment-related AEs was observed only in the training cohort (P = 0.017), and not in the validation cohort (P = 0.108). Given that immune-related AEs sometimes have a longer latent period, there might have been insufficient time in the present study for significant differences to emerge. Therefore, a longer follow-up time might be needed.

In summary, we have shown that changes in the plasma levels of CXCL2 and MMP2 were significantly associated with the clinical outcomes of anti-PD-1 therapy. Since these soluble immune mediators in plasma can be easily measured by minimally invasive blood sampling, they could be useful for monitoring of clinical outcomes in NSCLC patients receiving PD-1 inhibitor therapy. Nevertheless, our study had several limitations. One major weakness was that the number of patients studied was relatively small. Secondly, our study included patients with different clinical characteristics who received nivolumab or pembrolizumab, different lines of treatment, and different percentages of PD-L1 expression. These might cause the difference in results between the training and validation cohorts. Given the growing interest in immune checkpoint therapies for NSCLC patients, further large-scale studies of patients with similar characteristics are warranted to definitively confirm the usefulness of the markers we have identified.

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Figure legends

Figure 1.

(A) The schedule of treatment and peripheral blood sampling. (B) Association between changes in the plasma levels of CXCL2 and MMP2 and objective tumor responses. (C, D) Kaplan–Meier plots of progression-free survival and overall survival in the two groups divided according to the changes in the plasma levels of CXCL2 and MMP2 after treatment.

Figure 2.

(A) Time-dependent changes in the plasma levels of CXCL2 and MMP2. (B, C) (Top) Computed tomography (CT) images of a primary lesion before and after treatment in two representative patients (P3 and P25) showing remarkable anti-tumor responses. (Middle) Time-dependent changes in the radiographic response. (Bottom) Time-dependent changes in the plasma levels of CXCL2 and MMP2 from their baselines.

Figure 3.

(A) The treatment course of patient P49. Computed tomography (CT) images demonstrate that the patient developed pseudoprogression just after initiation of treatment with pembrolizumab. (B) The changes in the plasma levels of CXCL2 and MMP2 in P49. (C) Hematoxylin and eosin staining and immunohistochemical staining patterns for CD8, CXCL2 and MMP2 in tissue samples before and after pembrolizumab therapy.

Supplementary figure S1

Scatter plot of the changes in sum of longest tumor diameter versus those in the plasma levels of CXCL2 (A) and MMP2 (B). The statistical analysis was performed by Pearson product-moment correlation coefficient.

Supplementary figure S2

Kaplan–Meier plots of progression-free survival and overall survival for patients with and without treatment-related AEs in the training and validation cohorts. The differences were evaluated statistically by the log-rank test.

Figure 1

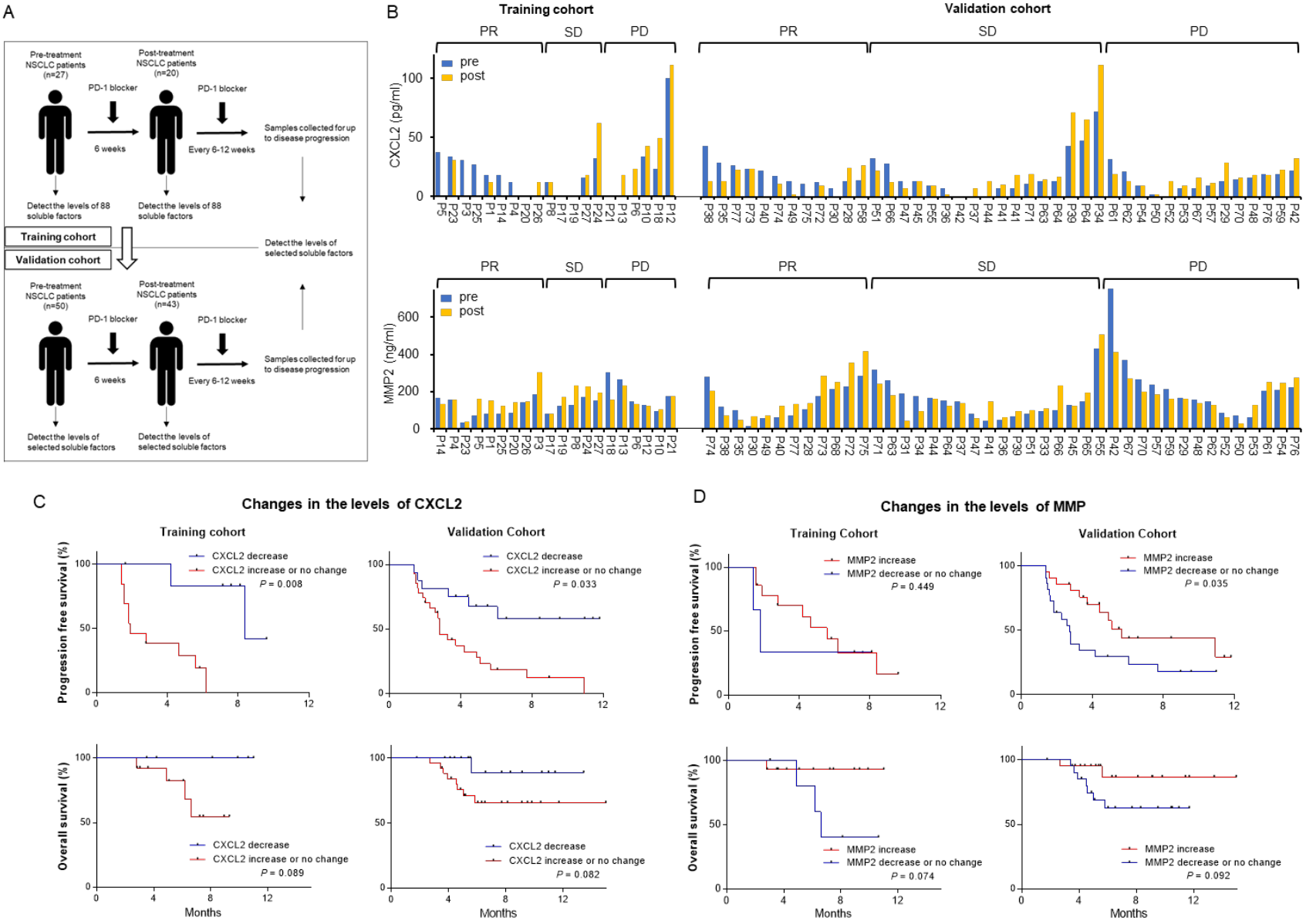

A
Pre-treatment NSCLC patients (n=27)
Post-treatment NSCLC patients (n=20)
PD-1 blocker
6 weeks
PD-1 blocker
Every 6-12 weeks
Samples collected for up to disease progression
Detect the levels of 88 soluble factors
Detect the levels of 88 soluble factors
Training cohort
Validation cohort
Detect the levels of selected soluble factors
Pre-treatment NSCLC patients (n=50)
Post-treatment NSCLC patients (n=43)
PD-1 blocker
6 weeks
PD-1 blocker
Every 6-12 weeks
Samples collected for up to disease progression
Detect the levels of selected soluble factors
Detect the levels of selected soluble factors
B
Training cohort
Validation cohort
PR
SD
PD
pre
post
CXCL2 (pg/ml)
MMP2 (ng/ml)
C
Changes in the levels of CXCL2
Training cohort
Validation Cohort
CXCL2 decrease
CXCL2 increase or no change
P = 0.008
P = 0.033
Progression free survival (%)
Overall survival (%)
P = 0.089
P = 0.082
Months
D
Changes in the levels of MMP
Training Cohort
Validation Cohort
MMP2 increase
MMP2 decrease or no change
P = 0.449
P = 0.035
P = 0.074
P = 0.092
Months

Figure 2

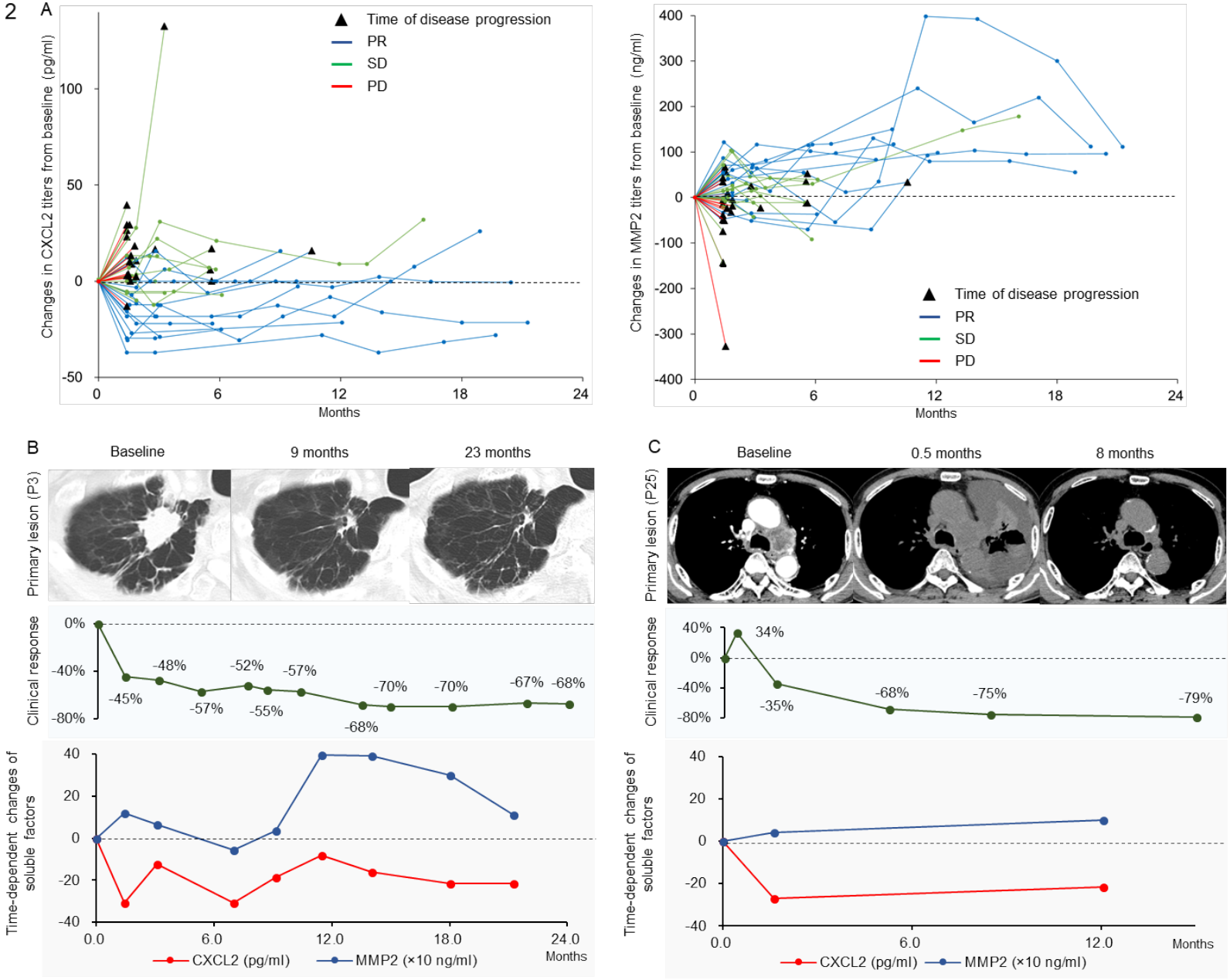

A
Time of disease progression
PR
SD
PD
Changes in CXCL2 titers from baseline (pg/ml)
100
50
0
-50
0
6
12
18
24
Months
Changes in MMP2 titers from baseline (ng/ml)
400
300
200
100
0
-100
-200
-300
-400
Time of disease progression
PR
SD
PD
Months
B
Baseline
9 months
23 months
Primary lesion (P3)
Clinical response
0%
-40%
-80%
-45%
-48%
-57%
-52%
-55%
-57%
-68%
-70%
-70%
-67%
-68%
Time-dependent changes of soluble factors
40
20
0
-20
-40
0.0
6.0
12.0
18.0
24.0
Months
CXCL2 (pg/ml)
MMP2 (×10 ng/ml)
C
Baseline
0.5 months
8 months
Primary lesion (P25)
Clinical response
40%
0%
-40%
-80%
34%
-35%
-68%
-75%
-79%
Time-dependent changes of soluble factors
40
20
0
-20
-40
0.0
6.0
12.0
Months
CXCL2 (pg/ml)
MMP2 (×10 ng/ml)

Figure 3

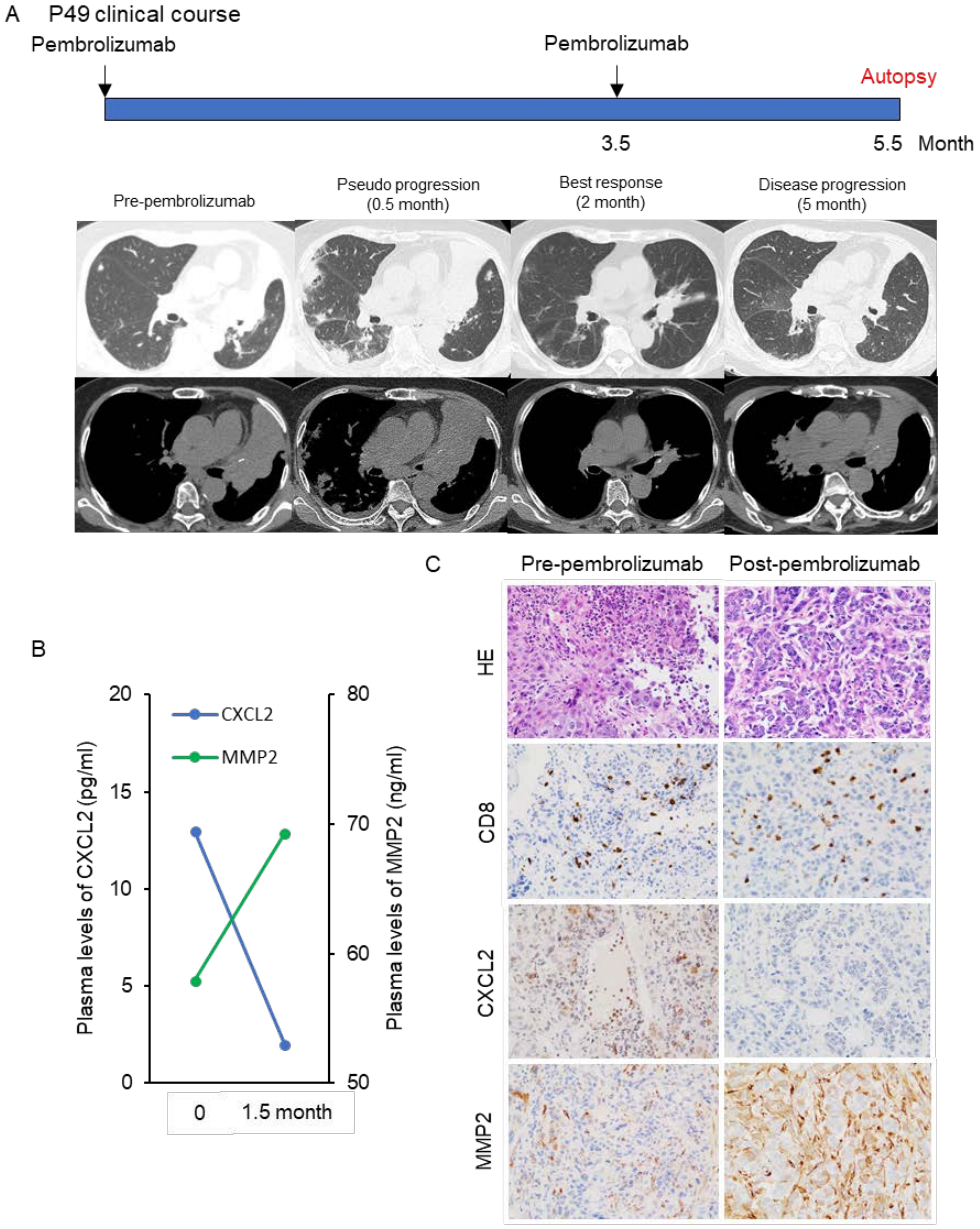
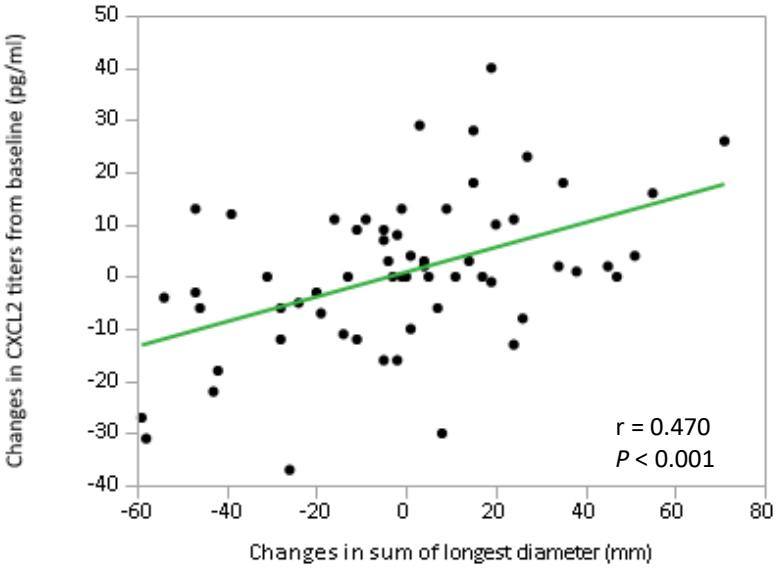

A P49 clinical course
Pembrolizumab
Pembrolizumab
Autopsy
3.5
5.5 Month
Pre-pembrolizumab
Pseudo progression (0.5 month)
Best response (2 month)
Disease progression (5 month)
B
CXCL2
MMP2
Plasma levels of CXCL2 (pg/ml)
Plasma levels of MMP2 (ng/ml)
20
15
10
5
0
80
70
60
50
0
1.5 month
C
Pre-pembrolizumab
Post-pembrolizumab
HE
CD8
CXCL2
MMP2

Figure S1

A


Changes in CXCL2 titers from baseline (pg/ml)
50
40
30
20
10
0
-10
-20
-30
-40
-60
-40
-20
0
20
40
60
80
Changes in sum of longest diameter (mm)
r = 0.470
P < 0.001

B

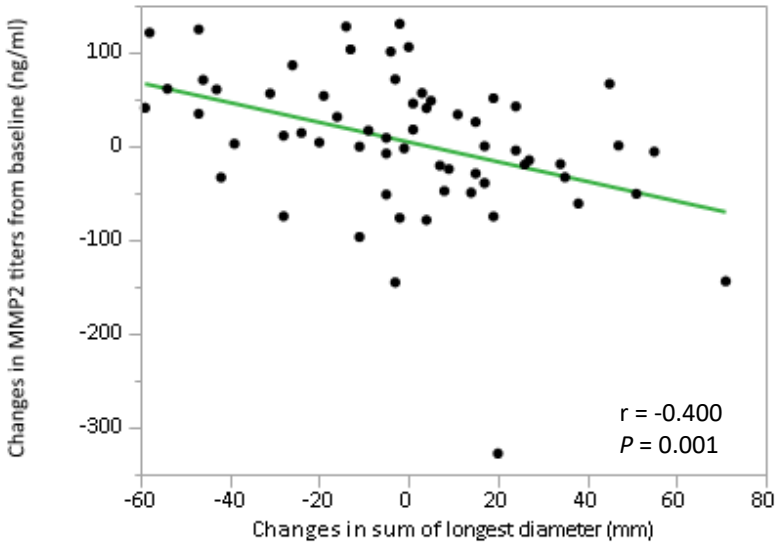

Changes in MMP2 titers from baseline (ng/ml)
100
0
-100
-200
-300
-60
-40
-20
0
20
40
60
80
Changes in sum of longest diameter (mm)
r = -0.400
P = 0.001

Figure S2

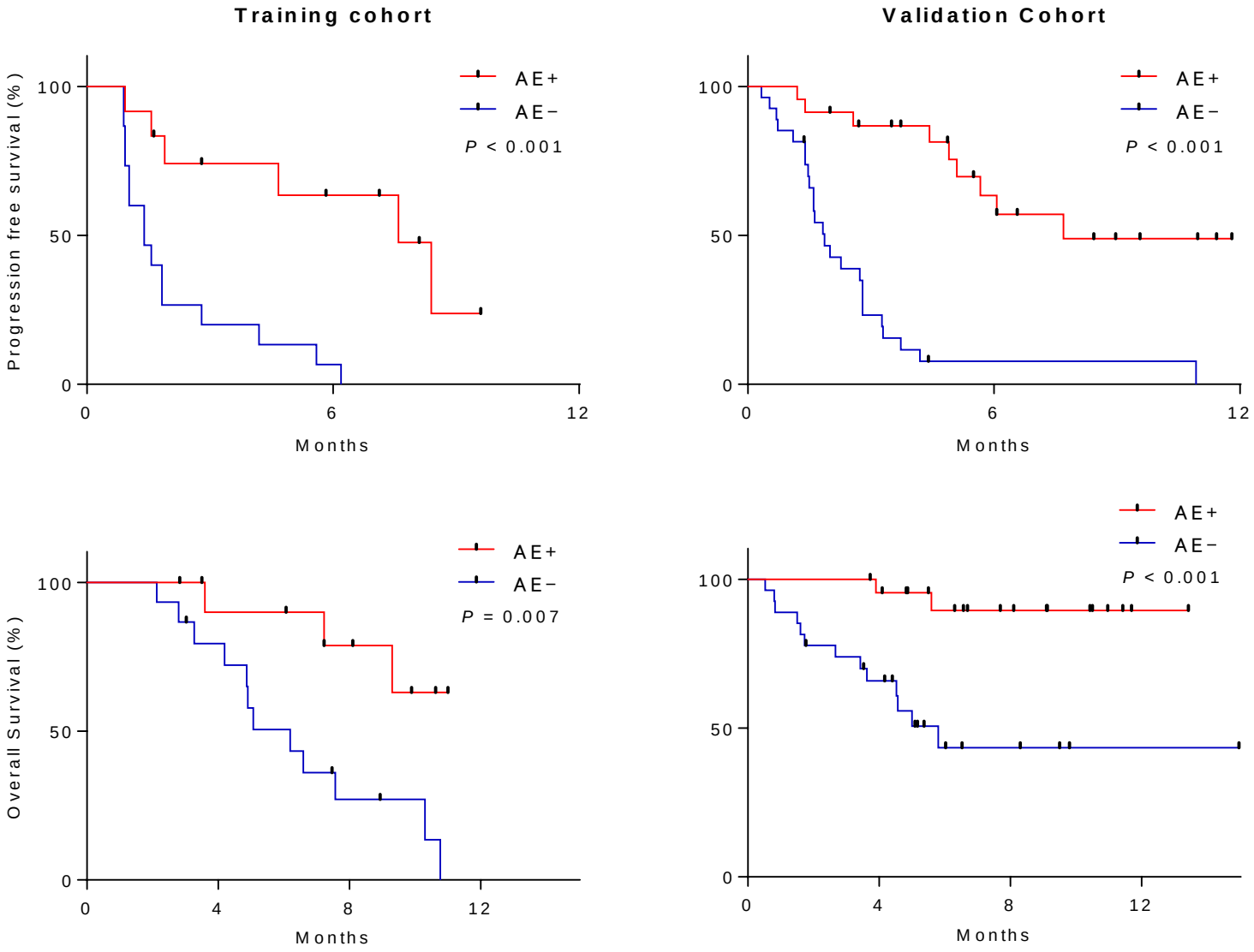

Training cohort
Validation Cohort
Progression free survival (%)
Overall Survival (%)
AE+
AE−
P < 0.001
P = 0.007
100
50
0
0
4
6
8
12
Months

Table 1. Characteristics of patients in the training and validation cohorts.

Variable	Number of patients (%)	
	Training (N = 27)	**Validation (N = 50)**
Age, years		
Median	69	67
Range	53-82	37-84
Gender		
Male	20 (74)	36 (72)
Female	7 (26)	14 (28)
PS		
0	7 (26)	26 (52)
1	10 (37)	13 (26)
2-3	10 (37)	11 (22)
Histology		
Adenocarcinoma	18 (67)	34 (68)
Squamous	7 (26)	7 (14)
Others	2 (7)	9 (18)
Driver mutation		
EGFR	7 (26)	8 (16)
ALK	1 (4)	1 (2)
Wild type	19 (70)	41 (82)
Smoking status		
Never smoker	7 (26)	11 (22)
Smoker	20 (74)	39 (78)
Stage		
Stage III	5 (19)	11 (22)
Stage IV or Recurrent	22 (81)	39 (78)
No. of prior systemic therapies		
0	0	16 (32)
1	10 (37)	22 (44)
2	7 (26)	5 (10)
3-7	10 (37)	7 (14)
PD-1 blocker		
Nivolumab	27 (100)	24 (48)
Pembrolizumab	0	26 (52)
PD-L1 expression on tumor cells (%)	(n = 19)	(n = 47)
0	11 (58)	7 (15)
1-49	4 (21)	13 (28)
50-100	4 (21)	27 (57)

Table 2. Association between soluble immune mediators in plasma and progression-free survival (PFS) or treatment-related adverse events (AEs).

	PFS				Treatment-related AEs			
Variable	P	HR	95% CI		P	OR	95% CI	
Training cohort								
Baseline mediators								
Chitinase 3-like-1	0.006	2.132	1.241	3.660	0.414	0.695	0.291	1.663
GM-CSF	0.005	0.407	0.217	0.763	0.030	2.843	1.109	7.286
Changes in mediators								
CXCL2	<0.001	4.57	1.889	11.06	0.017	0.079	0.010	0.633
VEGF	0.019	1.949	1.115	3.408	0.130	0.394	0.118	1.314
IFNα2	0.019	3.049	1.201	7.739	0.177	0.507	0.189	1.258
MMP2	0.012	0.429	0.222	0.828	0.053	3.213	0.987	10.457
Validation cohort								
Baseline mediators								
Chitinase 3-like-1	0.068	1.119	0.992	1.262	0.034	0.624	0.404	0.965
GM-CSF	ND	ND	ND	ND	ND	ND	ND	ND
Changes in mediators								
CXCL2	0.003	1.267	1.084	1.479	0.108	0.789	0.592	1.053
VEGF	0.852	1.012	0.896	1.142	0.290	0.926	0.803	1.068
IFNα2	0.962	0.994	0.777	1.271	0.878	1.025	0.745	1.411
MMP2	0.006	0.895	0.827	0.968	0.067	1.153	0.990	1.343

Abbreviations: PFS, progression-free survival; AEs, adverse events; HR, hazard ratio; CI, confidence interval
HR and OR in the training and validation cohorts were for a change of 1 standard error for each biomarker in the training cohort.

Table S1. Treatment-related adverse events (AEs).

	Training (N =27)		Validation (N =50)	
	Any	Grade 3 to 4	Any	Grade 3 to 4
	Number of patients with an event (%)			
Any event	12 (44)	3 (11)	24 (48)	6 (12)
General disorders				
Fatigue	2 (7)	0	5 (10)	0
Arthralgia	3 (11)	0	2 (4)	0
Skin				
Pruritus	4 (15)	0	3 (6)	0
Rash	3 (11)	0	5 (10)	1 (2)
Endocrine				
Hypothyroidism	2 (7)	0	2 (4)	1 (2)
Adrenal insufficiency	1 (4)	1 (4)	0	0
Hepatic				
AST increased	5 (19)	2 (7)	4 (8)	0
ALT increased	5 (19)	2 (7)	3 (6)	0
Pulmonary				
Pneumonitis	2 (7)	0	4 (8)	1 (2)
Gastrointestinal				
Diarrhea	1 (4)	0	5 (10)	0
Colitis	0	0	3 (6)	3 (6)
Renal				
Increased blood creatinine	1 (4)	0	1 (2)	0
Eye				
Uveitis	1 (4)	0	0	0

Table S2. Association between clinicopathological characteristics and progression-free survival (PFS) or treatment-related adverse events (AEs) in the training cohort.

Variable	PFS				Treatment-related AE			
	P	HR	95% CI		P	OR	95% CI	
Training cohort								
Patient characteristics								
Age (>65/<64 yr)	0.116	2.768	0.779	9.836	0.757	0.75	0.122	4.623
Gender (F/M)	0.561	1.333	0.507	3.506	0.334	0.400	0.062	2.568
PS	0.003	2.29	1.339	3.915	0.097	0.477	0.199	1.143
Histology (Sq/non Sq)	0.733	0.836	0.299	2.338	0.922	0.917	0.161	5.208
Smoking status (Smoker/none)	0.538	1.818	0.272	12.17	0.682	0.807	0.288	2.256
Stage (IV or Recurrent/III)	0.737	0.839	0.302	2.334	0.538	1.818	0.272	12.17
Number of prior systemic therapies (>3 /1, 2)	0.312	1.596	0.645	3.948	0.722	0.750	0.154	3.654
Complete blood count								
Neutrophil:lymphocyte ratio	0.672	0.895	0.536	1.495	0.852	0.928	0.426	2.026
Gene mutation testing								
Driver mutation (mutant/wild type)	0.999	1.001	0.359	2.787	0.707	1.375	0.262	7.220
Immunohistochemical staining								
PD-L1 expression on tumor cells (positive/negative)	0.331	0.578	0.192	1.743	0.845	1.200	0.194	7.441
Validation cohort								
Patient characteristics								
Age (>65/<64 yr)	0.814	0.921	0.464	1.829	0.357	1.714	0.545	5.396
Gender (F/M)	0.426	1.353	0.643	2.846	0.426	0.592	0.163	2.151
PS	0.018	2.472	1.172	5.314	0.130	0.321	0.074	1.396
Histology (Sq/non Sq)	0.418	1.482	0.571	3.844	0.085	0.145	0.016	1.308
Smoking status (Smoker/none)	0.315	0.675	0.314	1.452	0.386	1.842	0.464	7.319
Stage (IV or Recurrent/III)	0.140	1.702	0.840	3.451	0.097	0.368	0.113	1.198
Number of prior systemic therapies (≥4 /1-3)	0.973	0.982	0.345	2.796	0.603	1.533	0.306	7.690
Complete blood count								
Neutrophil:lymphocyte ratio	0.795	1.010	0.937	1.088	0.534	1.045	0.909	1.202
Gene mutation testing								
Driver mutation (mutant/wild type)	0.175	1.734	0.783	3.840	0.337	0.476	0.105	2.167
Immunohistochemical staining								

PD-L1 expression on tumor cells (positive/negative)	0.778	0.872	0.336	2.263	0.476	0.554	0.109	2.809

Abbreviations: PFS, progression-free survival; AEs, adverse events; HR, hazard ratio; CI, confidence interval; PS, performance status; Sq, squamous

Table S3. Association between changes in plasma CXCL2 and MMP2 levels and PD-L1 expression on tumor cells.

	Training			Validation		
		Patients with PD-L1 expression			Patients with PD-L1 expression	
Variable	**No**	**No. (%)**	***P***	**No**	**No. (%)**	***P***
CXCL2			0.266			0.387
Decrease	5	4 (80)		14	13 (93)	
Increase or no change	9	3 (33)		26	20 (77)	
MMP2			0.192			1.000
Increase	11	7 (65)		20	17 (85)	
Decrease or no change	3	0 (0)		20	16 (80)	