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Peripheral blood biomarkers associated with combination of immune checkpoint blockade plus chemotherapy in NSCLC

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Abstract.
BACKGROUND: Biomarkers predicting clinical outcomes of treating non-small cell lung cancer (NSCLC) with combination of immune checkpoint inhibitors (ICIs) and chemotherapy would be valuable.
OBJECTIVE: This study aims to seek predictors of combination of ICI/chemotherapy response in NSCLC patients using peripheral blood samples.
METHODS: Patients diagnosed with advanced NSCLC between July 2019 and May 2021 receiving combination of ICI/chemotherapy were included and assessed for partial responses (PR), stable disease (SD) or progressive disease (PD). We measured circulating immune cells, plasma cytokines and chemokines.
RESULTS: Nineteen patients were enrolled. The proportions of circulating natural killer (NK) cells within CD45$^+$ cells, programmed death 1 (PD-1)$^+$ Tim-3$^+$ T cells within CD4$^+$ cells, and the amount of chemokine C-X-C ligand (CXCL10) in the plasma were significantly elevated in PR relative to SD/PD patients (median 8.1%-vs-2.1%, $P = 0.0032$; median 1.2%-vs-0.3%, $P = 0.0050$; and median 122.6 pg/ml-vs-76.0 pg/ml, $P = 0.0125$, respectively). Patients with 2 or 3 elevated factors had longer progression-free survival than patients with 0 or only one (not reached-vs-5.6 months, $P = 0.0002$).
CONCLUSIONS: We conclude that NK cells, CD4$^+$ PD-1$^+$ Tim-3$^+$ T cells, and CXCL10 levels in pre-treatment peripheral blood may predict the efficacy of combination of ICI/chemotherapy in NSCLC.

Keywords: Combination of chemotherapy and immune checkpoint inhibitor, NSCLC, natural killer cells, CD4$^+$ cells, CXCL10

1. Introduction

Lung cancer is a common cancer causing 1.9 million deaths per year globally [1]. The introduction of immune checkpoint inhibitors (ICIs) has led to breakthroughs in the treatment of advanced non-small cell lung carcinoma (NSCLC). Based on the results of phase III trials, combined ICI/chemotherapy has become the current standard of care for the initial treatment of advanced NSCLC without driver mutations that can be targeted by small molecule inhibitors [2,3]. Patients with excellent responses to such ICI/chemotherapy combinations are expected to have long-term survival, but predicting in advance which patients will fall into this category is challenging. The availability of reliable biomarkers would be valuable in this context.

Several clinical trials that have indicated that the efficacy of ICI monotherapy for advanced NSCLC is as-

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sociated with the proportion of cells expressing programmed death ligand 1 (PD-L1) in the tumor tissue [4, 5]. ICIs are most effective in patients with high PD-L1 expression, with a reported 5-year survival rate of 31% [6]. This relationship between PD-L1 expression and clinical response has also been observed in combination of ICI/chemotherapy [2,3]. Consequently, the proportion of PD-L1 is used to predict the efficacy of ICIs in clinical practice. However, treatment strategies based on PD-L1 expression alone remain unsatisfactory; some patients with a PD-L1 high tumor proportion score (TPS) nonetheless fail to respond to ICIs, and some patients with low or negative TPS respond well to ICIs. In addition, there is a practical constraint in that the PD-L1 TPS cannot be evaluated in some patients due to the small amounts of tumor tissue available, or to having received only a cytological diagnosis. Therefore, in addition to PD-L1 expression analysis, non-invasive, reliable, and simple methods are warranted to predict the efficacy of ICI therapy.

Peripheral blood is easily accessible in a minimally invasive manner. Several previous studies have reported that CD8$^+$ T cells, CD4$^+$T cells, Natural Killer (NK) cells, and the neutrophil to lymphocyte ratio (NLR) in blood samples are useful for predicting the efficacy of ICIs in patients with lung cancer.

ICIs mediate their anti-tumor effects *inter alia* by blocking immune suppressive signals and preventing CD8$^+$ T cell exhaustion [7]. Hence, CD8$^+$ T cells have been well studied, not only in terms of quantity but also whether they are exhausted or activated and which subpopulations are present, all of which have been reported as predictors of response [8,9,10,11]. CD4$^+$ T cells and NK cells in the tumor microenvironment (TME) were also reported as predictors [9,12,13,14]. Several other predictors that have been reported are immune suppressive cells such as regulatory T cells (Tregs) [13] and myeloid-derived suppressor cells (MDSCs) [15, 16,17], cytokines and chemokines such as interleukin (IL)-6 [18], IL-8 [19,20] and chemokine C-X-C ligand 10 (CXCL10) [21]. The NLR and absolute counts of lymphocytes and monocytes can easily be measured in routine clinical practice [22,23,24].

However, the conclusions in these reports were based on data from patients who received ICI monotherapy, and some predictors have conflicting functions between ICI monotherapy and chemotherapy. Therefore, whether the same applies to combination of ICI/chemotherapy is unclear. Cytotoxic anticancer drugs affect the TME in multiple ways, including by reducing immune cell numbers due to bone marrow suppression or by activating immune cells via the release of damage-associated molecular patterns (DAMPs) [25]. It is therefore likely that predictors of responses to combination of ICI/chemotherapy and ICI monotherapy will differ. Despite advances in combination immunotherapy regimens, it is also unclear whether peripheral blood biomarkers can function as useful predictors for the success of such therapies. To this end, here we conducted a retrospective study to identify predictors of combination of ICI/chemotherapy response using pre-treatment peripheral blood samples.

2. Materials and methods

2.1. Patients

Patients diagnosed with unresectable locally advanced, postoperatively recurrent, or metastatic NSCLC at the Tohoku University Hospital between July 2019 and May 2021 were reviewed. Inclusion criteria for this study were age $\geqslant$ 20 years, histological confirmation of a diagnosis of NSCLC, administration of combination of ICIs/chemotherapy as first-line treatment, presence of written informed consent and existence of biobanked blood samples. Bevacizumab-containing regimens were also allowed as combination of ICIs/chemotherapy regimens in this study. Exclusion criteria were oncogenic driver mutation positivity, Eastern Cooperative Oncology Group Performance Status (ECOG-PS) $\geqslant$ 2, administration of corticosteroids or immunosuppressive drugs within the prior two weeks, or uncontrolled brain metastases. Postoperative recurrence was defined as cases of recurrence after surgery with curative-intent for resectable NSCLC. A history of adjuvant chemotherapy without ICI was allowed.

This study was approved by the institutional review boards of Tohoku University Hospital.

The medical records of patients were reviewed to extract clinical information, as follows: age, sex, ECOG PS, histology, clinical stage, PD-L1 expression by the tumor, complete blood count, first-line chemotherapy regimen, tumor response, and progression-free survival (PFS). A total of 19 patients was enrolled based on the above criteria.

All subjects were evaluated for tumor responses according to the Response Evaluation Criteria in Solid Tumors (RECIST 1.1). Patients were dichotomized into 1) Partial Response (PR) or 2) Stable Disease (SD)/Progressive Disease (PD) groups. PFS was defined as the time elapsed from the initiation of combi-

nation of ICI/chemotherapy until the first diagnosis of progressive disease, or death from any cause. Patients without documented clinical or radiographic disease progression or who were still alive were censored on the date of the last follow-up. The cutoff date of the study was March 31st, 2022.

2.2. Peripheral blood collection

Peripheral blood samples were collected the day before initiation and just before the third dose of the first-line treatment in all patients. Mononuclear cell preparation tubes (BD Vacutainer® CPT™) were employed to separate PBMCs from plasma. PBMCs were stored in liquid nitrogen, and plasma was stored at −80°C. We mainly used pre-treatment blood samples for the experiments. We used both pre- and post-blood samples only for measurement of DAMPs.

2.3. Flow cytometry

Cryopreserved PBMCs were thawed in a 37°C water bath and resuspended in PBS with 3% fetal bovine serum. Total cell numbers were counted, and 1×10^6 cells were plated per well in 96-well plates. Single-cell suspensions were stained with anti-human CD3-FITC (OKT3), CD14-PerCp-Cy5.5 (HCD14), HLA-DR-PE-Cy7 (L243), CD11b-APC (ICRF44), CD56-V421 (HCD56), CD45-PE (HI30), CD366 (Tim-3)-FITC (F38-2E2), CD4-PerCp-Cy5.5 (OKT4), CD279 (PD-1)-PE/Cy7 (EH12.2H7), CD45RA-APC (HI100), CD62L-BV421 (DREG-56), CD8-PE (SK1), CD25-BV421 (BC96), human IgG1, κ Isotype Ctrl- PE/Cy7 (MOPC-21), and human IgG1, κ Isotype Ctrl-FITC (MOPC-21). These antibodies were all purchased from BioLegend Japan (Tokyo, Japan). Foxp3-APC (FJK-16s) and Foxp3/transcription factor staining buffer were from Thermo Fisher Scientific (Tokyo, Japan). Intracellular staining was performed following the manufacturer's protocol. Dead cells were excluded by staining with Live/Dead NEAR IR (Thermo Fisher Scientific). Stained cells were analyzed on a FACSCanto II flow cytometer (BD Biosciences, Tokyo, Japan) using FACSDiva Software. We enumerated the proportions of circulating NK cells, T lymphocytes, Tregs, and MDSCs within the CD45+ population. Subpopulations of T cells and the expression of exhaustion markers were also assessed. Supplementary Fig. 1 shows the gating strategies.

2.4. Cytometric bead array

Concentrations of plasma Concentrations of plasma IL-4, IL-2, CXCL10, IL-1β, TNF-α, MCP-1, IL-17A, IL-6, IL-10, IFN-γ, IL-12p70, IL-8, and Free Active TGF-β1 were determined using the Cytometric Bead Array (CBA), following the manufacturer's protocol (BD Biosciences). All plasma cytokines were quantified by flow cytometry on a FACSCanto II flow cytometer (BD Biosciences) using FACSDiva and CBA software (BD Biosciences).

2.5. ELISA

Concentrations of plasma HMGB1 and calreticulin were measured using ELISA kits (Cloud-Clone Corp., Houston, USA), following the manufacturer's protocol. The concentration of these DAMPs was quantified by SpectraMax ABS (MOLECULAR DEVICES, Tokyo, Japan).

2.6. Statistical analysis

Statistical analyses were performed using the commercial software JMP (SAS Institute Japan, Tokyo, Japan) or GraphPad prism (GraphPad Software Inc. California, USA). Medians with 95% confidence intervals (CI) are given for most data. Differences between continuous variables were assessed using the Mann–Whitney U test. The Chi-square test was used to analyze categorical variables. Correlations were measured as Pearson correlation coefficients. PFS was evaluated with the Kaplan–Meier method, and differences between survival curves were evaluated using the log-rank test. Considering the exploratory approaches used, P-values were not corrected for multiple testing. A P-value < 0.05 was considered statistically significant.

3. Results

3.1. Patients' characteristics

The background characteristics of the patients are summarized in Table 1. Of the 19 patients 13 exhibited a PR and 6 SD/PD. The median age was 69 years (range, 52–78 years), and 14 (73.7%) were male. There were 13 (68.4%) adenocarcinomas and 6 (31.6%) squamous cell carcinomas. All patients received platinum-based chemotherapy together with ICIs, as follows: cisplatin + pemetrexed + pembrolizumab ($n = 4$), carboplatin + pemetrexed + pembrolizumab ($n = 8$), carboplatin + nab-paclitaxel + pembrolizumab ($n = 6$), and carboplatin + paclitaxel + atezolizumab + bevacizumab ($n = 1$). Two patients were at Stage IIIB,

Table 1
Patients' characteristics at baseline

Characteristics	Number (%) or Median (Range)			P Value
	ALL ($N = 19$)	PR ($N = 13$)	SD/PD ($N = 6$)	
Age	69 (52–78)	71 (52–78)	67 (63–77)	0.5346
Gender				
Male	14 (73.7)	9 (69.2)	5 (83.3)	0.5164
Female	5 (26.3)	4 (30.8)	1 (16.7)	
Performance status				0.1112
0	14 (73.7)	11 (84.6)	3 (50.0)	
1	5 (26.3)	2 (15.4)	3 (50.0)	
Histology				0.3421
Adenocarcinoma	13 (68.4)	8 (61.5)	5 (83.3)	
Squamous cell carcinoma	6 (31.6)	5 (38.5)	1 (16.7)	
Disease stage				0.5986
IVB	10 (52.6)	6 (46.2)	4 (66.7)	
IVA	5 (26.3)	4 (30.8)	1 (16.7)	
IIIB	2 (10.5)	2 (15.4)	0	
Postoperative recurrence	2 (10.5)	1 (7.7)	1 (16.7)	
PD-L1 TPS				0.2522
$< 1\%$	3 (15.8)	3 (23.1)	0	
1–49%	4 (21.1)	2 (15.4)	2 (33.3)	
$\geqslant 50\%$	9 (47.4)	7 (53.8)	2 (33.3)	
Unknown	3 (15.8)	1 (7.7)	2 (33.3)	
Regimen of ICI/chemotherapy				0.1927
CDDP+PEM+Pembro	4 (21.1)	1 (7.7)	3 (50.0)	
CBDCA+PEM+Pembro	8 (42.1)	6 (46.2)	2 (33.3)	
CBDCA+nab-PTX+Pembro	6 (31.6)	5 (38.4)	1 (16.7)	
CBDCA+PTX+Atezo+BEV	1 (5.3)	1 (7.7)	0	
Leukocyte count (/μl)				
Neutrophils	5320 (2420–10410)	5000 (2420–10410)	5770 (4810–7750)	0.1791
Lymphocytes	1380 (730–2570)	1360 (1120–2420)	1550 (730–2570)	0.9488
Monocytes	480 (250–1670)	480 (250–1670)	505 (440–920)	0.9487

TPS, tumor proportion score; CDDP, Cisplatin; PEM, Pemetrexed; CBDCA, Carboplatin; PTX, Paclitaxel; Pembro, Pembrolizumab; Atezo, Atezolizumab; BEV, Bevacizumab.

5 Stage IVA, 10 Stage IVB, and the remaining two had postoperative recurrence. None of the patients in this cohort received adjuvant chemotherapy. High PD-L1 expression was noted in 9 patients, and low or no expression in 4 and 3, respectively.

The median follow-up time was 14.8 months and median PFS of all patients was 7.4 months. The median PFS of patients with PR was 12.4 months, significantly longer those with SD/PD (4.6 months, $P = 0.0002$, Supplementary Fig. 2A). However, there was no significant difference in PFS between patients whose tumors exhibited a high level of PD-L1 expression vs. low or no expression (12.4 vs. 7.4 months, $P = 0.4469$, Supplementary Fig. 2B).

3.2. *NK cells and CD4$^+$ PD-1$^+$ Tim-3$^+$ T cells are associated with clinical efficacy*

In order to determine which if any blood immune cells correlated with clinical response, we compared the distribution of immune cell populations in PBMCs between the PR and the SD/PD groups. We found that the proportions of circulating NK cells and CD4$^+$ PD-1$^+$ Tim-3$^+$ T cells within the CD45$^+$ population were significantly higher in the PR group ($P = 0.0032$, and $P = 0.0050$ respectively, Fig. 1A, B). To identify whether these factors are potential biomarkers for predicting PFS, we dichotomized all patients into two groups based on the medians of NK cells (5.44%) or CD4$^+$ PD-1$^+$ Tim-3$^+$ T cells (2.12%) as shown in Fig. 1C, D, and compared PFS by the Kaplan–Meier method. We found that patients with $\geqslant$ median NK cells had a significantly longer PFS than those with lower NK cells (12.4 vs. 5.7 months, $P = 0.0411$, Fig. 1E). The same analysis was performed for CD4$^+$ PD-1$^+$ Tim-3$^+$ T cells. Patients with $\geqslant$ median percentages of these cells tended to have prolonged PFS (12.4 vs. 6.7 months, $P = 0.0551$, Fig. 1F). Univariate analysis suggested that NK cells and CD4$^+$ PD-1$^+$ Tim-3$^+$ cells were significant predictors of PFS (NK cells: HR 0.82, $P = 0.0067$. CD4$^+$ PD-1$^+$ Tim-3$^+$ T cells: HR 0.35, $P = 0.0309$). Patients' backgrounds were similar for all groups, except for the neutrophil count (Supplementary Tables 1, 2). NK cells remained significant in multi-

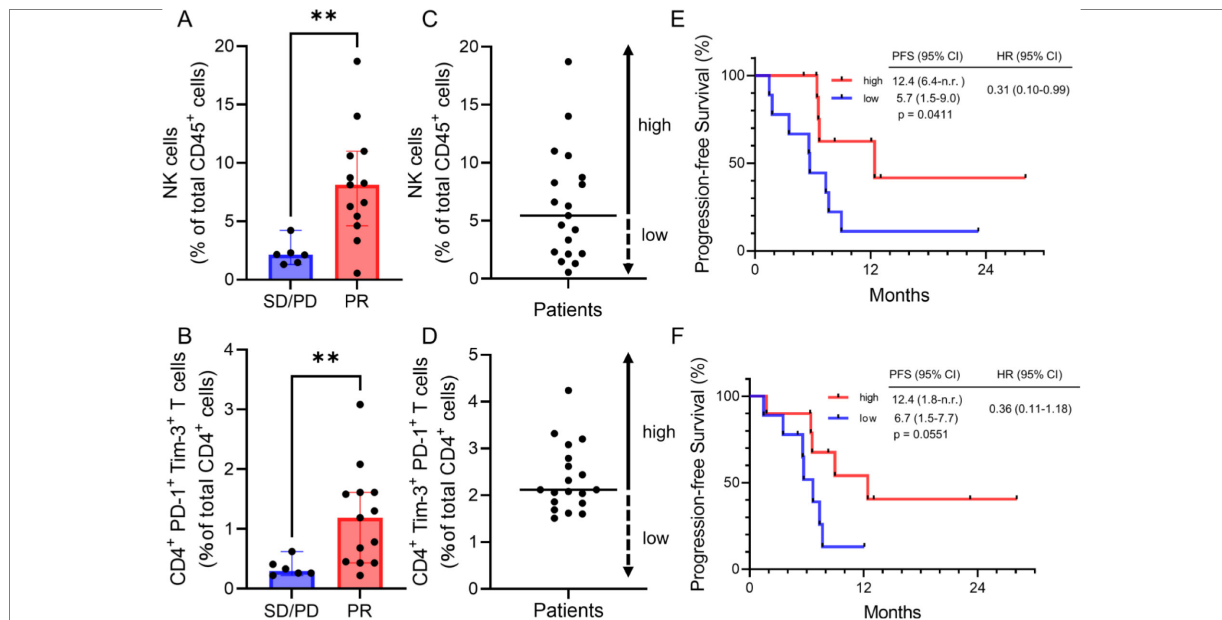

Fig. 1. Peripheral NK cells and CD4$^+$ PD-1$^+$Tim-3$^+$ cells predict clinical efficacy. (A, B) Percentages of NK cells within the CD45$^+$ population, and %CD4$^+$PD-1$^+$Tim-3$^+$ cells within the CD4$^+$ population in peripheral blood of patients with PR or SD/PD. Data are medians ± 95% CI. p-values were determined using the Mann–Whitney U test. (C, D) Dot plots of each patient's data. Patients were divided into higher and lower groups according to the median value (horizontal bar). (E, F) Kaplan-Meier curves of PFS for patients with higher and lower values of NK cells or CD4$^+$PD-1$^+$Tim-3$^+$ cells. $^{**}P < 0.01$; NK cells, natural killer cells; PFS, progression-free survival; PR, partial response; SD/PD, stable disease/progressive disease; HR, hazard ratio; CI, confidence interval.

variate analyses with neutrophil count as a covariate (HR 0.81, $P = 0.0059$) The proportions of circulating CD8$^+$ T lymphocytes, Tregs and MDSCs were also not significantly different between the PR and SD/PD groups (Supplementary Fig. 3).

3.3. *CXCL10 is associated with clinical efficacy*

In the TME, CD4$^+$ T cells help CD8$^+$ cytotoxic T cells via IL-2, as well as producing cytokines with direct anti-tumor activity, such as IFN-γ and TNFα [26]. NK cells also help CD8$^+$ T cells, are cytotoxic themselves, and additionally kill tumor cells via cytokines [27]. Because immune cells interact with each other via cytokines, we measured cytokine levels in plasma to seek correlations with the efficacy of combination therapy. Of the 13 cytokines tested in an essential immune response panel (see Materials and Methods), the titer of CXCL10 was significantly higher in the PR group ($P = 0.0125$, Fig. 2). Its median concentration was 122.6 pg/ml in the PR group, but only 76.0 pg/ml in the SD/PD group (Fig. 2A). As for the blood immune cells, we dichotomized all patients based on median values (Fig. 2B). This analysis showed that median PFS of patients with $\geqslant$ median CXCL10 (91.32 pg/ml) was prolonged relative to patients with low CXCL10 group (median PFS not reached vs. 5.7 months, $P = 0.0165$, Fig. 2C). Univariate analysis suggested that CXCL10 was a significant predictor of PFS (HR 0.97, $P = 0.0013$.). There was no significant difference in patients' backgrounds between those with higher or lower CXCL10 levels, except for age (Supplementary Table 3). CXCL10 remained significant in multivariate analyses with age as a covariate (HR 0.97, $P = 0.0154$) No cytokines other than CXCL10 were significantly different in PR and SD/PD patients (Fig. 3), although all cytokines tended to be higher in the PR group regardless of whether they are considered to be pro- or anti-tumorigenic.

3.4. *Plasma DAMPs are not correlated with clinical outcome*

DAMPs are released from dying cells or cells damaged by chemotherapy, and can activate several types of immune cells [28,29]. We hypothesized that DAMPs affect the efficacy of the combination of ICI/chemotherapy. We quantified HMGB1 and calreticulin in patients' plasma because they are DAMPs reported to be released by chemotherapy [30,31]. How-

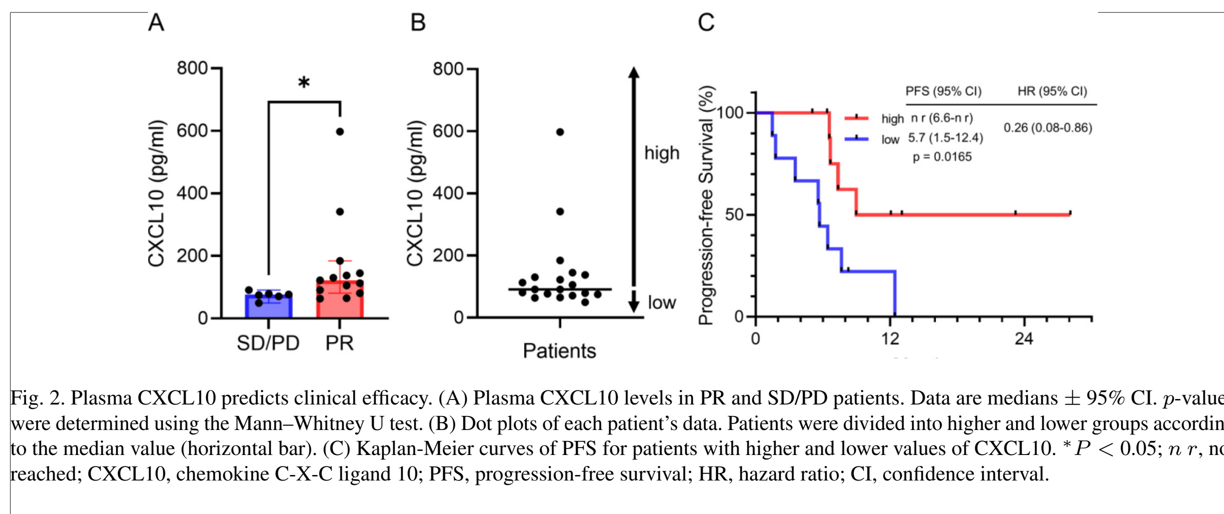

Fig. 2. Plasma CXCL10 predicts clinical efficacy. (A) Plasma CXCL10 levels in PR and SD/PD patients. Data are medians ± 95% CI. p-values were determined using the Mann–Whitney U test. (B) Dot plots of each patient's data. Patients were divided into higher and lower groups according to the median value (horizontal bar). (C) Kaplan-Meier curves of PFS for patients with higher and lower values of CXCL10. $^*P < 0.05$; $n\ r$, not reached; CXCL10, chemokine C-X-C ligand 10; PFS, progression-free survival; HR, hazard ratio; CI, confidence interval.

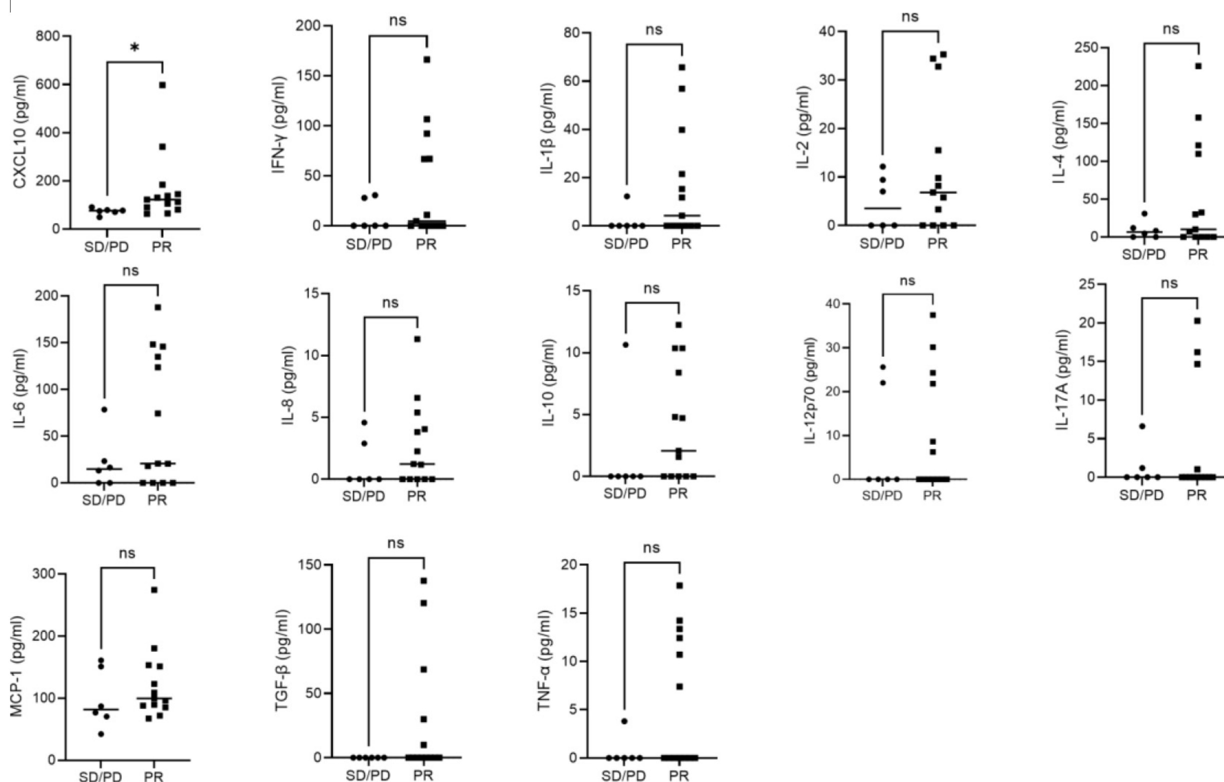

Fig. 3. Plasma cytokine concentrations. Plasma cytokines in PR and SD/PD patients. Dot plots are each patient's data and horizontal bars are medians. p-values were determined using the Mann–Whitney U test. $^*P < 0.05$; ns, not significant; PR, partial response; SD/PD, stable disease/progressive disease; CXCL, chemokine C-X-C ligand; IFN, interferon; IL, interleukin; MCP, monocyte chemotactic protein; TGF, transforming growth factor; TNF, tumor necrosis factor.

ever, there were no differences between PR and SD/PD patients for either of these DAMPs (Supplementary Fig. 4); neither were there any significant differences when comparing pre- and post-treatment. Therefore, we concluded that measuring these DAMPs is not useful for predicting treatment outcome.

3.5. *Prediction of the efficacy of combination immunotherapy*

Based on the above results, we concluded that 3 factors (NK cells, CD4$^+$ PD-1$^+$Tim-3$^+$T cells and CXCL10) could each predict the efficacy of combina-

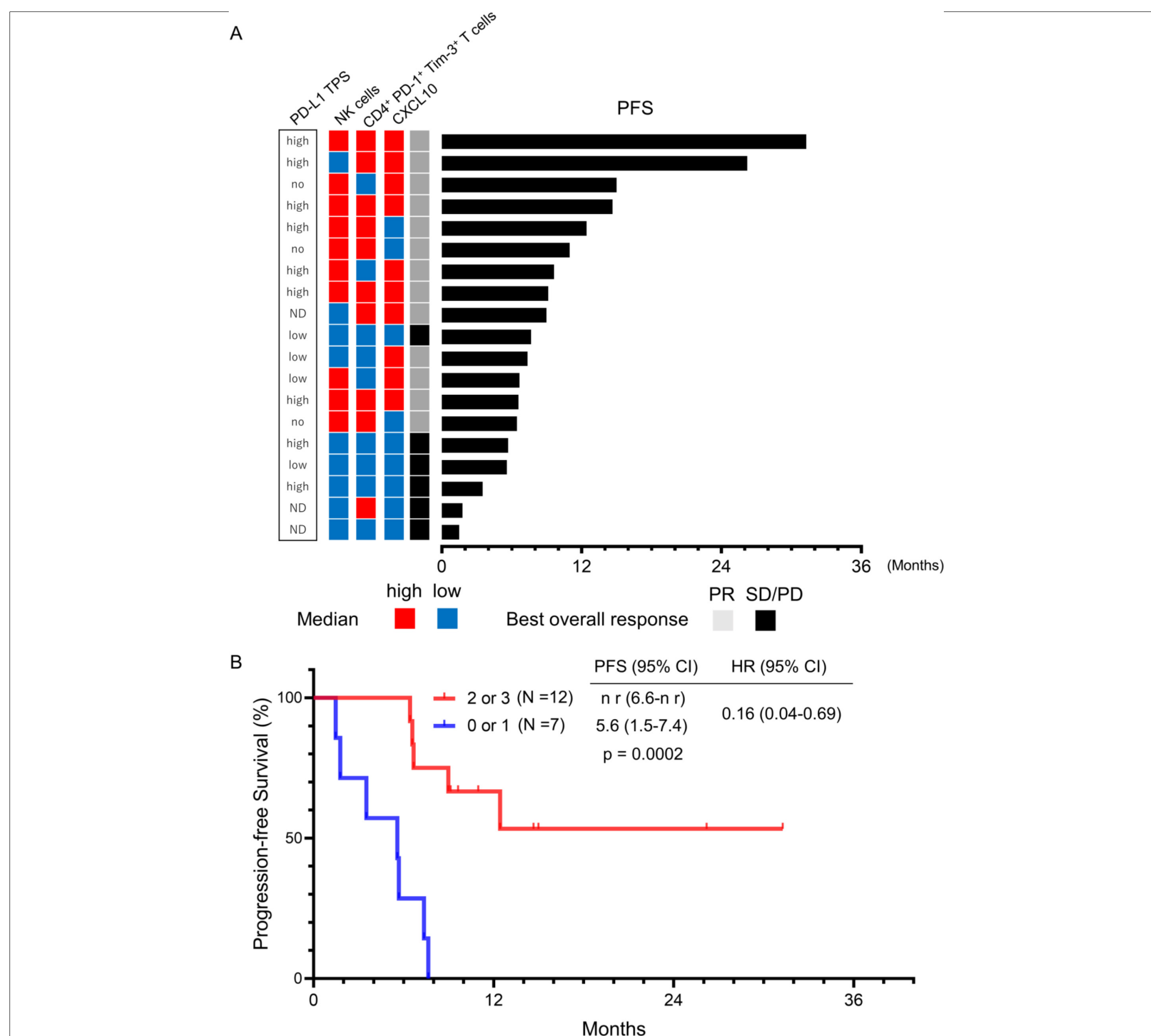

Fig. 4. Combinations of NK cells, CD4$^+$ PD-1$^+$Tim-3$^+$ T cells and CXCL10 are better predictors of ICI/chemotherapy outcome than any single factor. (A) Each patient is shown as a column, indicating the PD-L1 TPS, NK cells, CD4$^+$ PD-1$^+$Tim-3$^+$ T cells, CXCL10, best overall response, and PFS. (B) Kaplan-Meier curves of PFS for patients with two or three elevated factors among NK cells, CD4$^+$ PD-1$^+$ Tim-3$^+$, and CXCL10 versus those with zero or one factor. ICIs, immune checkpoint inhibitors; PD-L1, programmed death ligand 1; TPS, tumor proportion score; NK cells, natural killer cells; CD4$^+$Tex, exhausted T cells (CD4$^+$ PD-1$^+$Tim-3$^+$ T cells); CXCL10, chemokine C-X-C ligand 10; PFS, progression-free survival; PR, partial response; SD/PD, stable disease/progressive disease; HR, hazard ratio; CI, confidence interval; n r, not reached, ND; no data.

tion of ICI/chemotherapy. We then investigated whether these factors were correlated with each other by means of Pearson correlation coefficient estimates (Supplementary Fig. 5) no significant correlation was found among them. Figure 4 shows the impact of PD-L1 TPS, the proportions of NK cells and CD4$^+$ PD-1$^+$ Tim-3$^+$ T cells, and the level of CXCL10 on response to treatment and on PFS. Patients with higher levels of two or all three of these 3 factors exhibited a higher PR rate (X^2 (1) $= 15.03$, $p = 0.0001$), superior responses to treatment and longer PFS than patients with zero or only one factor (median PFS not reached vs. 5.6 months, $P = 0.0002$, Fig. 4).

4. Discussion

In this study, we sought peripheral blood biomarkers informative for the outcome of combination of ICI/chemotherapy in patients with NSCLC. The main

finding was that patients experiencing a partial response had higher percentages of NK cells and CD4$^+$ PD-1$^+$ Tim-3$^+$ T cells and a higher CXCL10 level in the peripheral blood than patients with stable or progressive disease. In addition, higher levels of NK cells, CD4$^+$ PD-1$^+$ Tim-3$^+$ T cells and CXCL10 predicted a better PFS, and a combination of these biomarkers further improved the accuracy of prediction. In contrast, PD-L1 TPS failed to predict treatment efficacy in this study.

To the best of our knowledge, only two studies have previously reported predictors for combination of ICI/chemotherapy in NSCLC patients [32,33]. Miriam et al reported that elevation of NK cells in peripheral blood was a favorable predictive factor, consistent with our study. In the TME, NK cells play important roles such as preventing metastasis, attracting different immune cells into the tumor and directly lysing cancer cells that are resistant to ICI-facilitated CD8$^+$ T cell cytotoxicity due to lack of expression MHC class I molecules [34,35,36,37].

Patients with higher proportion of CD4$^+$PD-1$^+$Tim-3$^+$ T cells showed better clinical responses in our study. We hypothesized that PD-1 and Tim-3 are markers of exhaustion of CD4$^+$ as well as CD8$^+$ T cells and thus are targets for ICIs, such that patients with higher levels of these cells have a better clinical response to blockade of these checkpoints. In a previous study of patients not receiving immunotherapy, those with high CD4$^+$ PD-1$^+$ Tim3^{high} T cells were reported to have a worse prognosis [38]. This is consistent with exhaustion of these cells and their potential susceptibility to checkpoint blockade. However, exhaustion of CD4$^+$ T cells have not been fully uncovered. We used PD-1 and Tim-3 as markers of exhaustion because they are the most commonly-used markers of exhaustion of CD8$^+$ T cells [39], and it was reported that CD4$^+$ T cells with reduced effector functions upregulate Tim-3 and PD-1, paralleling phenotypes observed in exhausted CD8$^+$T cells [40]. However, after completing our analysis, a study was published suggesting that CD39 rather than Tim-3 should be used to assess exhaustion of CD4$^+$ T cells [41]. Therefore, the function of PD-1$^+$ Tim-3$^+$ CD4$^+$ T cells requires further clarification in order to determine the role of these cells in the TME and to assess which markers are most appropriate for assessing exhaustion status.

Of the 13 cytokines we analyzed, only CXCL10 was significantly elevated in the PR group relative to the SD/PD group, and patients with higher CXCL10 had longer PFS. Several studies reported that CXCL10 induced migration of immune cells into the tumor and that this correlated with clinical efficacy [42,43]. Thus, high levels of CXCL10 in the peripheral blood may indicate active immune cell infiltration into tumor tissue also in combination therapy.

As mentioned, we hypothesized that changes in TME by chemotherapy might alter predictors between ICI monotherapy and combination of ICI/chemotherapy. For the ICI monotherapy, NK cells, CD4$^+$PD-1$^+$ T cells, and CXCL10 were reported as good predictors of response [14,21,44]. On the other hand, for the chemotherapy, NK cells and CXCL10 did not have function as predictors [45,46], and CD4$^+$ PD-1$^+$ T cells were reported as a poor predictor [38,47]. Although further studies are needed, these results may suggest that predictors of ICI monotherapy are also useful to predict combination of ICI/chemotherapy.

Our study has several limitations. First, it was retrospective with only a small number of patients. To validate our results, we searched for publicly-available data on PBMC from patients who received ICIs plus chemotherapy. However, there appear to be no such cohorts with information on treatment and response. In addition considering that our biomarker analysis was exploratory, we did not correct P-values for multiple cytokine testing. Therefore a prospective study with a larger number of cases is needed to validate the results of this study. Second, we selected CBA as the cytokine assay method in order quantify multiple cytokines simultaneously using only a small sample volume. However, CBA is less sensitive than ELISA, and some cytokines may have been undetectable because of their low concentration. Nonetheless, this does not detract from the finding that CXCL10 is a strong predictor of efficacy at the concentration range detectable by the CBA technique. Lastly, our study could not evaluate the local TME. Most of the patients in this study were diagnosed by bronchoscopy. Because tissue samples obtained by transbronchial lung biopsy or transbronchial needle aspiration were small and crushed, it was difficult to evaluate the local TME and compare with results of blood samples.

In conclusion, this study revealed that NK cells, CD4 PD-1$^+$ Tim-3$^+$ T cells, and CXCL10 in pre-treatment peripheral blood may predict the efficacy of combination of ICI/chemotherapy in NSCLC. Because the TME is very complex due to the variety of immune cells and cytokines involved, the elevation of two or more of these three markers may be a more useful predictive biomarker in clinical practice than any single factor. A further prospective large-scale multi-center study is warranted to validate these results.

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For every author, his or her contribution to the manuscript needs to be provided using the following categories:

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Supervision: Eisaku Miyauchi, Hisatoshi Sugiura.

Supplementary data

The supplementary files are available to download from http://dx.doi.org/10.3233/CBM-230301.

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