Lung Cancer 132 (2019) 107–113

Contents lists available at ScienceDirect

Lung Cancer

journal homepage: www.elsevier.com/locate/lungcan

Predictive value of serum protein levels in patients with advanced non-small cell lung cancer treated with nivolumab

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ARTICLE INFO

JEL classifications:
Preclinical and translational research

Keywords:
Non-small-cell lung cancer
Immune checkpoint inhibitors
Noninvasive diagnostics
Immune related adverse events
Serum biomarker

ABSTRACT

Background: Although programmed cell death-ligand-1 (PD-L1) expression in tumor tissue has been established as predictive biomarker for the anti-programmed cell death-1 (PD-1) antibody treatment of non-small-cell lung cancer (NSCLC), additional biomarkers are critically needed. We evaluated serum proteins relevant to immune checkpoint blockade in patients with NSCLC treated with nivolumab to identify novel non-invasive predictive biomarkers.

Patients and methods: Patients with advanced NSCLC, who had failed at least one prior chemotherapy regimen, received nivolumab monotherapy (3 mg/kg, Q2W) until progressive disease (PD) or unacceptable toxicity was observed. Blood samples were collected at baseline and week 4. Fifty-seven serum protein levels were quantified with a Milliplex MAP assay. The associations of both clinical benefit (CB) and the onset of immune related adverse events (irAEs) with serum proteins levels were evaluated.

Results: Thirty-eight patients with advanced NSCLC were enrolled in the study, with 38 and 32 paired serum samples at baseline and week 4 being available for efficacy analysis and irAE analysis, respectively. In durable CB (DCB) patients compared with non-DCB patients, the baseline serum levels of BMP-9 were significantly higher, whereas the follistatin, IL-8, IP-10, and TNF-α levels were significantly lower. In irAE patients compared with non-irAE patients the serum levels of G-CSF and RANTES at week 4 were significantly higher, whereas the levels of leptin were significantly lower. A multivariate analysis revealed that follistatin and IP-10 were statistically associated with DCB ($p < 0.05$) and RANTES was associated with irAE onset ($p < 0.05$). In a subset of irAE-developed patients, RANTES levels decreased after steroid administration, supporting its involvement in irAE.

Conclusion: Serum proteins have the potential to be predictive markers for DCB and irAEs onset in patients with NSCLC treated with nivolumab. In addition, antitumor activity and irAEs may not be regulated by the same mechanisms.

1. Introduction

Lung cancer is a leading cause of cancer-related deaths worldwide [1]. Approximately 85% of patients with lung cancer are diagnosed with non-small cell lung cancer (NSCLC). Because two-thirds of the patients show local advancement or have metastatic tumors at diagnosis, chemotherapy is the major strategy for the treatment of lung cancer. Molecular targeted therapies for the treatment of lung cancer have developed remarkably in the past two decades and therapies that target driver mutations, such as EGFR mutations and ALK protein fusions, are the major treatment strategies for patients with NSCLC. Among these drugs, immune checkpoint inhibitors (ICIs) have prompted a paradigm shift in the treatment of various malignancies including NSCLC [2–6].

Programmed cell death-1 (PD-1) is an important immune checkpoint molecule. It is expressed on cytotoxic T lymphocytes and binds to

Abbreviations: PD-1, programmed cell death 1; PD-L1, programmed cell death-ligand 1; NSCLC, non-small-cell lung cancer; DCB, durable clinical benefit; PR, partial response; SD, stable disease; PD, progressive disease; irAE, immune related adverse event; TMB, tumor mutational burden

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https://doi.org/10.1016/j.lungcan.2019.03.020
Received 25 January 2019; Received in revised form 19 March 2019; Accepted 20 March 2019

its ligand programmed cell death ligand 1 (PD-L1), which is expressed on tumor cells. The PD-1/PD-L1 co-stimulation between cytotoxic T lymphocytes and tumor cells results in immune suppression by tumor cells. Anti PD-1/PD-L1 antibodies such as nivolumab, pembrolizumab, and atezolizumab inhibit the association between PD-1 and PD-L1 and prevent the escape of tumor cells from the immune system [7]. Although recent clinical studies have demonstrated that PD-L1 expression on tumor cells is associated with clinical benefits in the treatment of NSCLC [3–5], anti PD-1/PD-L1 immunotherapy is also effective in some patients whose PD-L1 levels are low in their tumor tissue [2,4]. Some reports have also suggested that tumor mutational burden (TMB), as well as the neoantigen burden and the presence of tissue infiltrating lymphocytes are predictive biomarkers in treatment with ICIs [8–10]. Although these biomarkers predict clinical benefits in cancer treatment, the cost and complex methodologies they require to accurately assess these biomarker levels present formidable challenges to their adoption for general use in the clinic. Therefore, it is imperative to identify more specific and sensitive biomarkers to identify patients that are likely to respond to treatment with ICIs. Recently, liquid biopsy, a lowly-invasive method, has been intensively used to discover novel biomarkers. For example, it has been suggested that the neutrophil to lymphocyte ratio and the absolute blood cell count are predictive biomarkers in treatment with ICIs [11,12].

Treatment with ICIs is often accompanied by severe adverse events, referred to as irAEs, such as rash, pneumonitis, hepatitis, autoimmune diabetes, and colitis [13]. There have been some reports that show a correlation between clinical benefits and the onset of irAE in NSCLC [14–16]. Because some irAE cases could be lethal, it is very important to be able to predict their onset. However, there are currently no biomarkers that can predict the onset or grade of irAEs.

In this study, we explored the biomarkers associated with clinical benefits such as tumor response and onset of irAE. Using a multiplex quantitative protein assay, we evaluated the serum levels of proteins consisting of cytokines, chemokines, angiogenic factors, and growth factors in patients with NSCLC treated with nivolumab and evaluated their association with tumor response and the onset of irAEs.

2. Materials and methods

2.1. Study design

Patients with advanced NSCLC who had failed at least one prior chemotherapy regimen received nivolumab monotherapy (3 mg/kg, Q2W) until progressive disease (PD) or unacceptable toxicity were observed. Blood samples were collected serially at baseline, and at week 4, week 8, week 12, and PD. Tumor responses were classified into partial response (PR), stable disease (SD), or progressive disease (PD) based on RECIST v1.1. In addition to the response defined by RECIST, efficacy was also evaluated by durable clinical benefit (DCB; PR and SD that lasted for more than 6 months) or non-DCB. The exclusion criteria were as follows; (1) patients who received administration of systemic corticosteroid; (2) patients who were transferred to other hospitals; (3) patients who died during the treatment; (4) patients whose disease progressed. When patients were treated with systemic corticosteroids, they were excluded from the following analysis time point.

2.2. Sample collection

Blood samples were collected in a serum separation tube (Venoject II-Autosep, Terumo Corp., Tokyo, Japan). These samples were incubated at room temperature for 30 min and centrifuged at 1160 × *g* for 10 min. The collected serum was aliquoted and frozen at -80 °C until use. All samples were processed within one hour.

2.3. Milliplex assay

Serum proteins were quantified by Milliplex MAP assay using human cytokine/chemokine panel 1, human angiogenesis/ growth factor panel 1 and a multi-species TGF-β panel (Millipore, Billerica, MA). Assays were performed according to the manufacturer's instruction. Standards or serum samples were mixed with antibody-bound beads, which were chemically dyed, and incubated overnight at 4 °C. Beads were washed and then incubated with the biotinylated detection antibody for one hour at room temperature. After washing, the beads were incubated with phycoerythrin-labeled streptavidin for thirty minutes at room temperature and the median fluorescent intensities were quantified with a Luminex 200 analyzer (Luminex, Austin, TX). All samples were measured in duplicate.

2.4. Immunohistochemistry

Immunohistochemistry of PD-L1 was performed as reported previously [15]. Formalin-fixed paraffin-embedded tissue of 4-μm thickness was stained with an anti-human PD-L1 rabbit monoclonal IgG antibody (ab205921, clone 28-8 from Abcam, Cambridge, MA, USA). Two independent lung pathologists evaluated the expression of PD-L1 in tumor cells. Briefly, tissues including 100 or more tumor cells were regarded as evaluable and cells with any staining intensity on the membrane at expression levels of 0%, 1–49%, and 50% or higher in a section were recorded.

2.5. Ethics

This study was approved by the Institutional Review Boards at Wakayama medical university and with the University Medical Hospital Information Network (UMIN) Clinical Trials Registry under the identifier UMIN000024414. All participants provided written informed consent.

2.6. Statistical analysis

All statistical analyses were carried out using JMP Pro software ver. 13.0 (SAS Institute, Cary, NC, USA). A Mann-Whitney U test was applied to assess the association between serum protein levels and clinical benefit. Statistically significant variables were further evaluated with a multivariate logistic regression analysis. A Spearman's test was applied to assess the correlation between serum protein levels and progression free survival (PFS). Cut-off values of serum protein levels were estimated with a receiver operating characteristic (ROC) curve. Time to event analysis was performed with Kaplan-Meier methods and log rank test. A p value of < 0.05 was considered as significant.

3. Results

3.1. Patients

A total of thirty-eight patients were registered between January 2016 and March 2017. The patient characteristics are outlined in Table 1. The characteristics of the patients were as follows: median age 68.5 (range, 49–86); male/female, 28/10; smoker/never smoker, 26/12; non-squamous/squamous/others, 25/11/2; previous treatments 0/1/≥2, 1/17/20; performance status of 0/1/≥2, 8/25/5; Stage III/IV, 11/27. The objective response rate was 27% (9/38) and the disease control rate was 50% (19/38). Finally, all patients and 32 patients were included in the efficacy and irAE analysis at baseline, respectively (Fig. 1).

3.2. Detection of multiple serum protein levels

In this study, we evaluated the levels of 57 serum proteins consisting

Table 1
Patient characteristics.

	N = 38
Age	
Median (range)	68.5 (49–86)
Sex-n(%)	
Male	28 (74)
Smoking history-n(%)	
Smoker	26 (68)
Histology-n(%)	
Squamous cell carcinoma	11 (29)
Non-squamous cell carcinoma	27 (71)
EGFR mutant	6
ALK fusion	1
Previous treatment-n(%)	
0–1	18 (47)
≥2	20 (53)
ECOG PS-n(%)	
0–1	33 (87)
≥2	5 (13)
C-stage-n(%)	
III	11 (29)
IV	27 (71)
Best response	
PR	9 (24)
SD	10 (26)
PD	19 (50)
PD-L1 expression (%)	
≥50%	8(21)
1–49%	13(34)
0%	0 (0)
Unknown	17(45)
Objective response rate-%	
	22%

of cytokines, chemokines, angiogenesis factors, and growth factors (Supplementary Table 1). Among these, 23 proteins were excluded from further analysis because their levels were not within acceptable thresholds. Therefore, in total we investigated 34 proteins.

3.3. Correlation between serum protein levels and clinical benefit

To explore biomarkers predictive of a clinical benefit from nivolumab treatment, we evaluated the association between serum protein levels and clinical benefits in patients with advanced NSCLC treated with nivolumab. We first evaluated differences in the serum protein levels between DCB and non-DCB patients. Between these, we found that the baseline levels of five serum proteins, BMP-9, follistatin, IL-8, IP-10, and TNF-α were all significantly different by a univariate analysis (Table 2, Supplementary Fig. 1A-F). With BMP-9, follistatin, IL-8, IP-10, and TNF-α as covariates, we performed multivariate logistic regression analysis. The multivariate analysis showed that baseline follistatin and IP-10 levels were significantly associated with DCB (Table 2, Fig. 2A, B). We also determined the optimal cut-off value with a receiver operating characteristic (ROC) curve to distinguish between DCB and non-DCB and found that the area under the curve was 0.74 for follistatin and 0.81 for IP-10, respectively (Supplementary Fig.2A, B). Based on the cut-off values as stratification points, we further evaluated the potential of follistatin and IP-10 as predictive biomarkers. PFS was significantly longer in patients with low follistatin levels (median PFS, not reached; 95% CI, 36 days-not reached) than in those with high follistatin levels (median PFS, 59 days; 95% CI, 40–127 days; log-rank p = 0.026) (Fig. 2C). Similar results were observed between IP-10-low group (median PFS, 322 days; 95% CI, 48 days-not reached) and IP-10-

Table 2
Univariate analysis and multivariate analysis of proteins associated with DCB and irAE.

	DCB			
	Univariate	Multivariate		
	p value	Odds Ratio	OR (95% CI)	p value
BMP-9	0.0124	1.004	0.9866–1.029	0.6284
Follistatin	0.0299	0.9966	0.9922–0.9997	0.0300
IL-8	0.0212	0.9628	0.8944–1.013	0.1543
IP-10	0.0046	0.9941	0.9838–0.9997	0.0369
TNF-α	0.0123	1.075	0.7952–1.459	0.6227
	irAE			
	Univariate	Multivariate		
	p value	Odds Ratio	OR (95% CI)	p value
G-CSF	0.0415	1.015	0.9843–1.054	0.3346
Leptin	0.0344	0.9998	0.9994–1.000	0.0692
RANTES	0.0465	1.000	1.000009–1.0001	0.0105

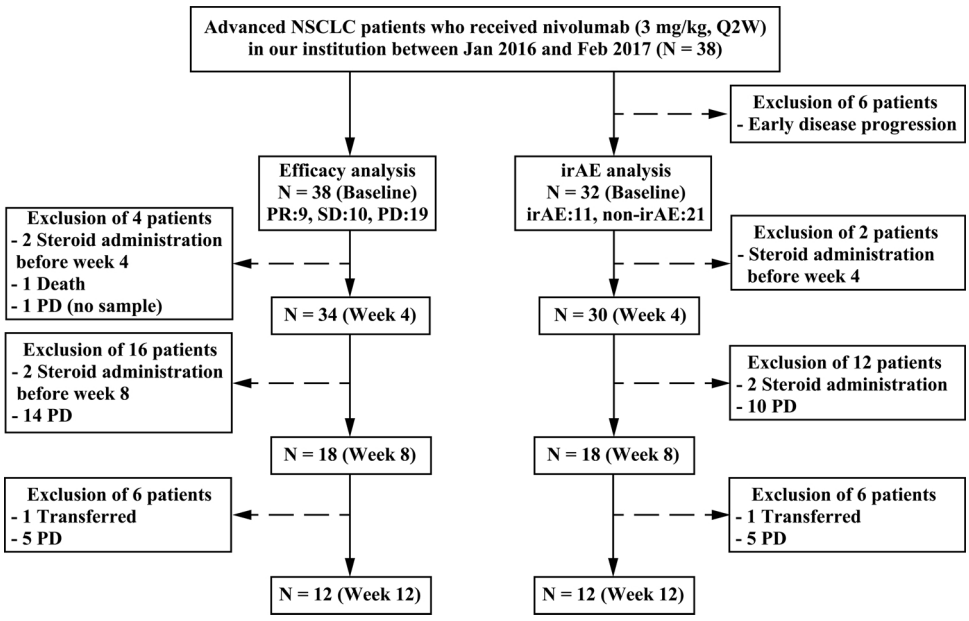

Fig. 1. Flow diagram outlining the study.

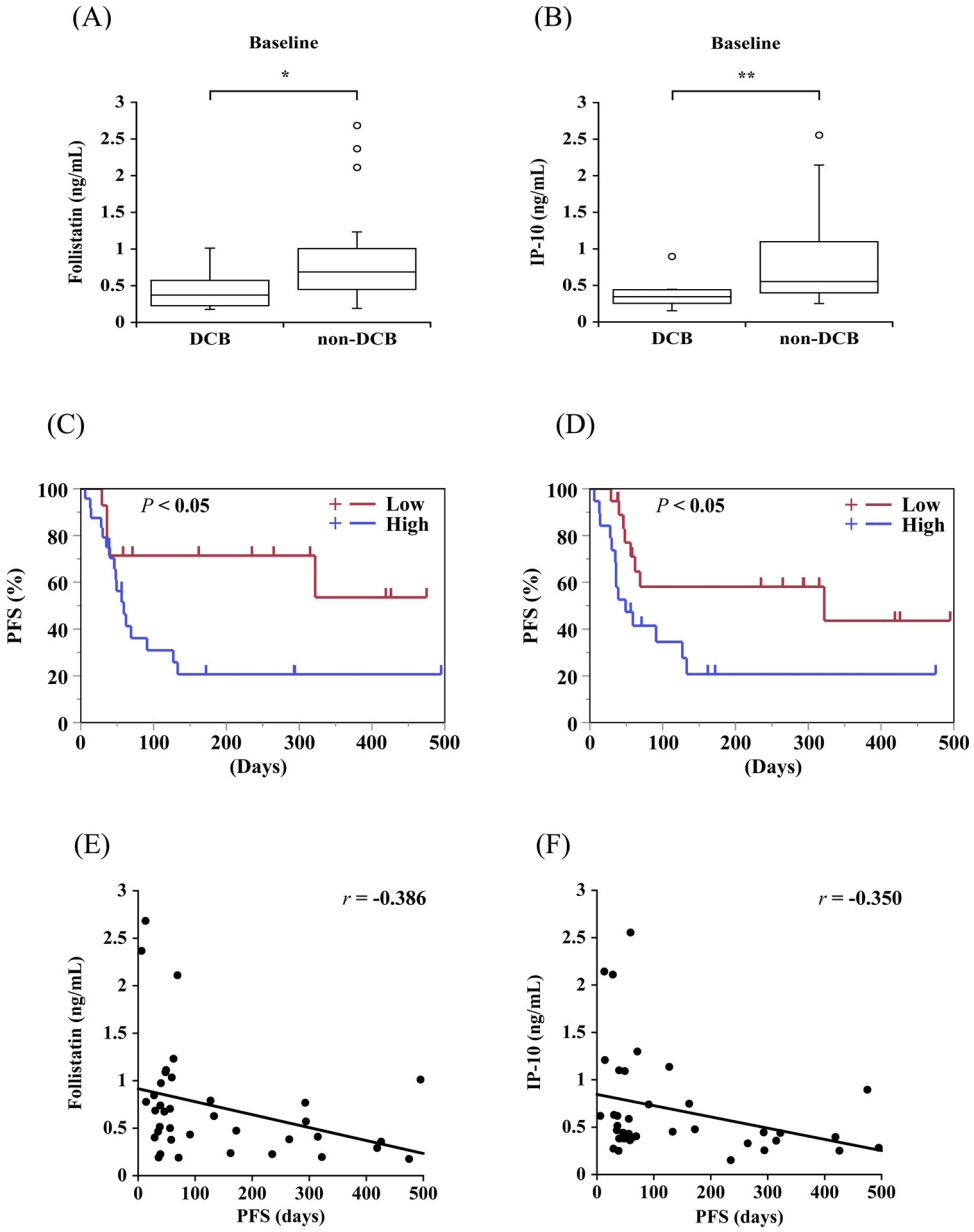

Fig. 2. Serum proteins levels are significantly associated with DCB.
Serum protein levels were assessed between DCB and non DCB patients. Box and whisker plots of baseline follistatin (A) and IP-10 levels (B). Kaplan-Meier curves of PFS stratified by the cut off value of ROC curves in follistatin (C) and IP-10 (D). Correlations between PFS and follistatin (E) and PFS and IP-10 (F). *p < 0.05, **p < 0.01.

high group (median PFS, 49 days; 95% CI, 30–127 days, log-rank p = 0.029) (Fig. 2D). These protein levels were also associated with a longer PFS in each entire cohort (Fig. 2E, F). These data further suggest that follistatin and IP-10 could be potential biomarkers to predict a patient subset who are likely to benefit from nivolumab treatment. Although IL-8 has previously been reported to have a predictive potential [17], it was not identified by the multivariate analysis in this study, only showing a weak correlation with a longer PFS (Supplementary Fig. 1B, 1E). No significant changes were observed in any of the proteins measured between baseline and week 4 between DCB and non-DCB (data not shown).

We also evaluated whether the proteins levels were correlated with the PD-L1 expression levels in tumor tissue. However, no significant correlation was observed between serum protein levels and PD-L1 levels in tumor tissue (data not shown).

3.4. Proteins associated with irAE onset

Next, we explored the use of serum biomarkers to predict the risk or occurrence of irAEs. Although no significant differences were observed between irAE and non-irAE patients at baseline, three proteins, G-CSF, leptin, and RANTES were significantly different between irAE and non-irAE patients at week 4 (Table 2, Supplementary Fig. 3 A, B). With G-CSF, leptin and RANTES as covariates, a multivariate analysis revealed that only the levels of RANTES were significantly associated with irAE onset (Table 2, Fig. 3A). In addition, we tracked the time-course of changes in RANTES levels in irAE patients. Eleven patients developed irAE, and of those seven were administrated corticosteroid. We were able to obtain paired serum samples before and after steroid administration from four patients (Fig. 3B). After steroid administration, the RANTES levels decreased in all four patients (Fig. 3C). These data suggest that RANTES is involved in the onset of irAEs caused by

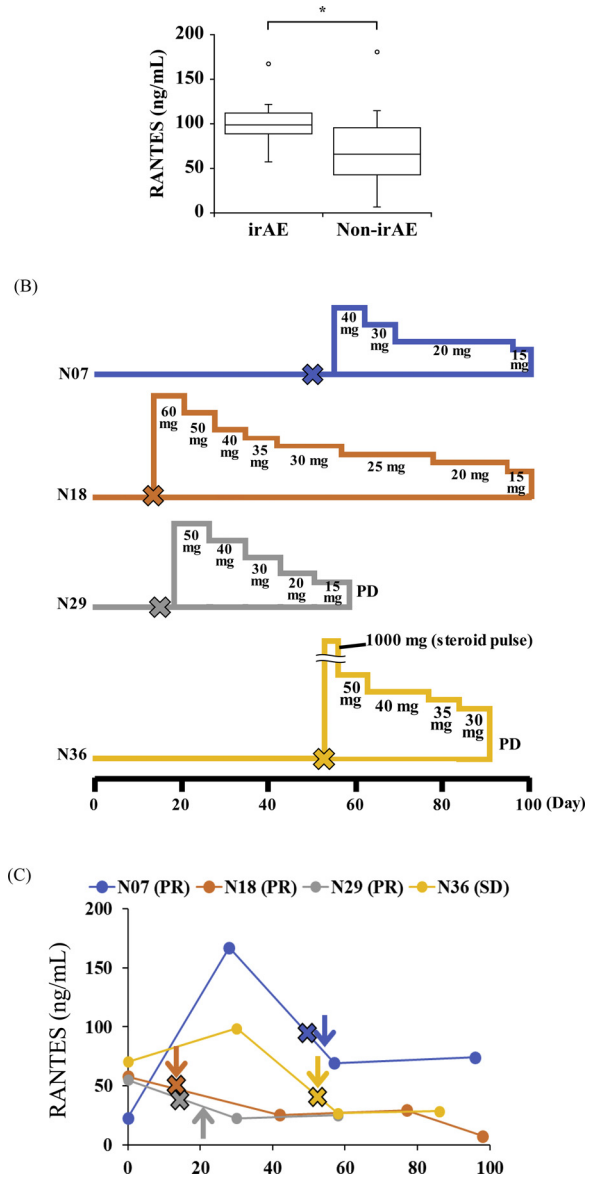

Fig. 3. Association between serum protein levels and irAEs.
Serum protein levels were assessed between irAE and non-irAE patients at week 4. (A) Box and whisker plot of RANTES. (B) Schedule showing the corticosteroid treatment in each patient. The closed crosses indicate the date of irAE onset. (C) Time-course analysis of serum protein levels in steroid-administrated patients. Changes in serum RATNES levels. The closed crosses indicate the date of irAE onset. The arrowheads indicate the date of steroid-administration. *p < 0.05.

nivolumab treatment.

4. Discussion

PD-1/PD-L1 blockade elicits unprecedent clinical benefits in patients with NSCLC. PD-L1 expression on tumor cells is an established biomarker for the efficacy of ICIs. However, despite implementing PD-L1 companion diagnostics, a considerable subset of patients still do not benefit from treatment with ICIs. Therefore, establishing other biomarkers is an urgent task needed to precisely select patients who are more likely to benefit from treatment with ICIs. In the present study, we explored the serum proteins associated with clinical outcome and the onset of irAEs in patients with NSCLC treated with nivolumab. A multivariate analysis revealed that the baseline levels of follistatin and IP-10 were significantly associated with DCB at baseline. Both of these were also associated with longer PFS. We also found that RANTES at week 4 was associated with the onset of irAEs.

Tumor cells regulate the immune suppressive microenvironment by recruiting immune cells such as Tregs, myeloid derived suppressor cells (MDSCs), and M2 macrophages [18]. Inflammatory cytokines and chemokines secreted from tumor cells recruit these immune cells into the tumor tissue, suggesting a relevant role for serum proteins in the efficacy of ICIs [17,19,20]. Follistatin is a secreted protein that is ubiquitously expressed in humans. Follistatin functions as a potent activin and bone morphogenetic protein (BMP) binding protein thereby antagonizing these proteins [21]. Recent data has also revealed that serum follistatin levels are associated with both lung cancer and prognosis. Serum follistatin levels have been shown to be significantly higher in patients with lung cancer compared with both healthy controls and patients with benign lung diseases [22]. Moreover, follistatin inhibits lung cancer cells from activin-A-induced apoptosis, and higher serum follistatin levels are associated with a poor prognosis [23]. Whereas the fact that follistatin levels correlate with tumor progression and prognosis is known, little is known about the function of follistatin in the ICI treatment of NSCLC. Given the nature of ICIs, there is a possibility that follistatin participates in creating an immune suppressive microenvironment to suppress antitumor T cell activation or promote the accumulation of immune suppressive cells. Human $CD4^+$ T cells express BMP-2, -4 and -6 after activation with CD3/CD28 antibodies *in vitro*. Moreover, autocrine BMP can be inhibited by a receptor-antagonizing antibody or a receptor kinase inhibitor thereby suppressing the proliferation of $CD4^+$ T cells [24]. These data support our hypothesis that follistatin could suppress $CD4^+$ T cells by antagonizing BMP signaling, resulting in a clinical benefit from nivolumab treatment (Fig. 4).

IP-10/CXCL10 is an inflammatory chemokine that is produced by various cells including leukocytes and acts as a potent chemoattractant for T cells. IP-10 has been reported to create an immune suppressive microenvironment mobilizing immune suppressive cells such as regulatory T cells (Tregs) [25,26]. It has been reported that NSCLC cells express IP10 [27,28], thus Tregs may be attracted by the action of IP-10 derived from tumor cells followed by the creation of an immune suppressive microenvironment (Fig. 4). From our data, no correlations were observed between follistatin and IP-10 levels and little has been reported on the association between follistatin and IP-10. The crosstalk between follistatin and IP-10 should be investigated to further clarify

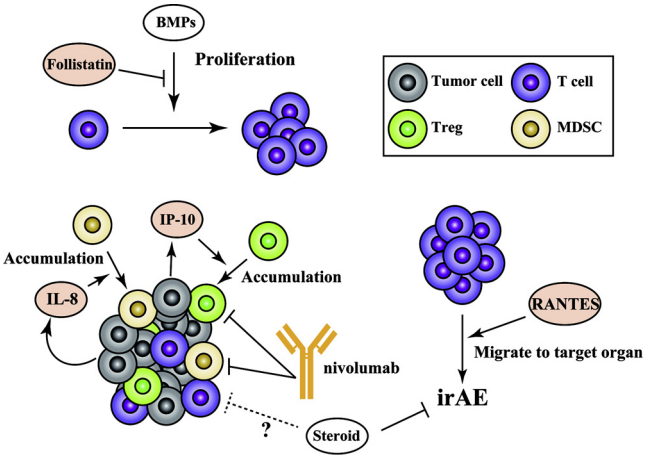

Fig. 4. Schematic model of the role of follistatin, IP-10, IL-8, and RANTES in tumor response and irAEs.
Follistatin induces the formation of Tregs from naïve T cells and IP-10 then recruits these Tregs. Tumor-derived IL-8 attracts the MDSCs to the tumor site. Nivolumab releases tumor cells from the immune suppressive microenvironment. After immune activation RANTES causes the accumulation of T cells in lymphoid tissue and they become activated. Although corticosteroid can suppress RANTES levels the antitumor activity is not blocked.

the mechanisms of clinical benefit from nivolumab treatment in patients with lung cancer.

Sanmamed et al. reported that changes in IL-8 levels in responders could predict responsiveness to anti-PD-1 treatment in NSCLC and melanoma [17]. In the present study, a similar trend was observed in terms of tumor response and durable clinical benefit (Supplementary Fig. 1E and Fig. 4), but this was not confirmed by a multivariate analysis (Table 2). A recent study has shown that an IL-8 blocking antibody decreased MDSCs in the tumor tissue of an experimental breast cancer xenograft model [29]. Given that tumor derived IL-8 attracts MDSCs and that intra-tumor IL-8 levels are associated with the accumulation of MDSCs, IL-8 could directly control the establishment of the tumor immune microenvironment [30,31]. Therefore, serum IL-8 levels may have the potential to identify patients that are likely to benefit from nivolumab treatment in advanced NSCLC and so should be further investigated.

The detailed mechanisms of irAEs still remain elusive [13]. One of the mechanisms was reported by Amarnath et al. using a mouse model of graft-versus host disease (GVHD) where they reported that Tregs mediate immune suppression *in vivo* by modulating the PD-1 pathway [32]. We observed the potential involvement of RANTES in irAE caused by nivolumab treatment in patients with NSCLC and some reports have shown that RANTES is one of the serum proteins relevant to the development of GVHD [33–35]. RANTES functions as a chemoattractant of a variety of immune cells such as activated T cells and a knockout of the CCL5 receptor reduces both the severity of both systemic and target organ GVHD [35], supporting our findings that RANTES may be a potential biomarker for the onset of irAE (Fig. 4). Therefore, surveillance of these proteins levels may be helpful in predicting the onset of irAE. However, we did not find any difference in the baseline protein levels except at week 4. This indicates that it is not possible to predict irAE onset before initiation of nivolumab treatment and that careful monitoring of the serum protein levels during treatment will be required. It is, however, still beneficial to identify patients who are more likely to develop irAEs and this should be further studied in a larger cohort.

Several studies have reported that the onset of irAEs is correlated with the efficacy of nivolumab treatment in patients with advanced NSCLC [14–16]. We expected that there would be some overlapping proteins between efficacy and irAE onset. However, we did not identify any overlapping proteins associated with both nivolumab efficacy and irAE onset. Therefore, there is a possibility that the efficacy and irAEs induced by nivolumab may be regulated differently in a spatiotemporal manner (Fig. 4). Indeed, one of the patients who developed irAE- was administered nivolumab only once and was treated with corticosteroid immediately after irAE diagnosis. Although the patient was continuously under immune-suppression, the tumor response was maintained even after corticosteroid treatment. It has been reported that steroid use for the management of irAEs does not affect the efficacy of ipilimumab in melanoma patients [36]. On the other hand, it has been reported that baseline steroid levels negatively affect the efficacy of PD-1/PD-L1 blockade in patients with NSCLC [37]. Little is known about the association of mechanisms between the tumor response and irAEs induced by nivolumab and this needs to be further investigated.

There are limitations in the present study. The first is that this study is an exploratory study and the size of the study cohort is small. We are now recruiting NSCLC patients treated with pembrolizumab as in-house study cohort. Moreover, we plan to analyze the serum samples from a prospective multicenter study in NSCLC patients treated with atezolizumab (UMIN000033133), which will serve as a large validation cohort [38]. The second is that the follow-up period where clinical data is available is relatively short and we need to evaluate the significance of these serum markers in terms of long-term clinical benefit. The third is that we did not include PD-L1 expression levels as a covariate in multivariate analysis because we did not observe statistical significance in univariate analysis (Supplementary Fig. 5). However, the small sample size may have affected the results of statistical analysis on PD-L1 expression and we should address this in the future study.

In conclusion, we have found that the baseline serum levels of IP-10 and follistatin could be potential biomarkers associated with clinical benefit and that RANTES levels at week 4 are associated with the onset of irAE in patients with advanced NSCLC treated with nivolumab. Further clinical investigation is warranted to confirm these findings and to develop these serum markers as predictive biomarkers for the treatment of NSCLC with ICIs.

Conflict of interest

Yasuhiro Koh, Hiroaki Akamatsu and Nobuyuki Yamamoto have received research funding and honoraria from Bristol-Myers Squibb and Ono Pharmaceuticals Co. Ltd. The remaining authors declare no conflict of interest.

Funding sources

This study was supported by Project for Cancer Research and Therapeutic Evolution (P-CREATE) from the Japan Agency for Medical Research and Development, AMED grant numbers 16cm0106418h0001 and 17cm0106418h0002 (Y.K. and N.Y.), and JSPS KAKENHI grant number JP16K18430 (J.O.).

Acknowledgements

We thank all the patients who participated in this study. We thank Drs. Junya Fukuoka and Kentaro Hayashi for PD-L1 immunohistochemistry and evaluation.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.lungcan.2019.03.020.

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