Original Article

DOI: 10.5582/bst.2018.01158

Prognostic factors of daily blood examination for advanced melanoma patients treated with nivolumab

Hisako Okuhira, Yuki Yamamoto*, Yutaka Inaba, Kayo Kunimoto, Naoya Mikita, Takaharu Ikeda, Chikako Kaminaka, Yoshimi Minami, Nobuo Kanazawa, Fukumi Furukawa, Masatoshi Jinnin

Department of Dermatology, Wakayama Medical University, Wakayama, Japan.

Summary **Biomarkers to distinguish patients with advanced melanoma responsive to nivolumab are of great interest. Therefore, we examined the possibility that laboratory data of daily blood examination become novel biomarkers. Laboratory data of 16 melanoma patients who were treated with nivolumab were retrospectively analyzed. Patients were classified as responder group or non-responder group. Examined were: white blood cell count (WBC), absolute lymphocyte counts (ALC), absolute neutrophil count (ANC), absolute monocyte count (AMC), absolute eosinophil count (AEC), and absolute basophil count (ABC), as well as levels of lactate dehydrogenase (LDH), C-reactive protein (CRP), one hour value of erythrocyte sedimentation rate (ESR), and 5-S-cysteinydopa (5-S-CD). Responder group showed significantly higher baseline levels of ESR or CRP and significantly lower ALC level before nivolumab treatment. Additionally, nivolumab treatment decreased the levels of CRP, ESR, and ANC, while it increased ALC level in the responder group. CRP was the most effective in distinguishing responder group from non-responder group both before and during treatment, according to the receiver operating characteristic (ROC) curve. We firstly showed that ESR is also the baseline biomarker of the efficacy of nivolumab. Furthermore, we confirmed that CRP is useful to predict the efficacy both before and during the treatment, and suggested that CRP is the most effective biomarker among daily blood examination by using ROC curve analysis. There is a possibility that nivolumab treatment may be more effective for malignant melanoma with stronger inflammation.**

Keywords: Absolute lymphocyte counts, absolute neutrophil counts, biomarker, malignant melanoma, PD-1

1. Introduction

Until recently, dacarbazine chemotherapy was mainly used for treatment of advanced malignant melanoma. Its effect was limited; however, the five-year survival rate was around 10% (*1*). In 2014, anti-programmed cell death protein 1 (PD-1) monoclonal antibody, nivolumab, was released in Japan, ahead of other countries. PD-1 is an immunosuppressive receptor belonging to the CD28 family and expressed on activated T cells to suppress T cell proliferation and effector function (*2*). An immune checkpoint, PD-1 is thought to play an important role in controlling adaptive immune response under physiological conditions (*3*). Nivolumab has been utilized for the treatment of advanced malignant melanoma and improves median overall patient survival. According to NCCN guideline version 2018, the combination of anti-PD-1 antibodies (nivolumab/pembrolizumab) or immunocheckpoint inhibitors (nivolumab/ipilimumab) is recommended as the first line immunotherapy for metastatic/unresectable melanoma. Nivolumab has also become available for non-small cell lung carcinoma (*4,5*), renal cell carcinoma (*6*), Hodgkin's lymphoma (*7*), squamous cell carcinoma of the head and

Released online in J-STAGE as advance publication August 29, 2018.

**Address correspondence to:*
Dr. Yuki Yamamoto, Department of Dermatology, Wakayama Medical University, 811-1 Kimiidera, Wakayama 641-0012, Japan.
E-mail: yukiy@wakayama-med.ac.jp

neck (*8*), and will be used for treatment of additional cancers in the future.

On the other hand, in patients with advanced stage melanoma, objective response rates of nivolumab were 43.7% and median progression-free survival was 6.9 months (*9*). Overall mortality and recurrence rates, however, remain high in the patients treated with immune checkpoint inhibitors. There is great interest in identifying biomarkers that will allow the selection of patients who would respond to nivolumab because of the associated substantial side effects and treatment costs. We therefore examine the possibility of daily blood examination laboratory data as new biomarkers.

2. Materials and Methods

2.1. *Patient material*

Enrolled in this study were sixteen patients with unresectable malignant melanoma, who were treated with nivolumab in Department of Dermatology, Wakayama Medical University, between September 2014 and March 2017. Excluded were patients with single nivolumab treatment and those with inflammatory diseases, such as rheumatoid arthritis, which could affect laboratory data. Nivolumab dose was fixed at 2 mg/kg every three weeks in 13 patients or 3 mg/kg every two weeks in the remaining three patients.

Patient characteristics are summarized in Table 1. Mean age was 71.3 years (range, 48-87), and 43.8% (n = 7) were male. Performance status (PS) was 0 to 1 in 93.8% (n = 15) of patients, while 56.3% (n = 9) of patients had stage IV disease. According to Curtin classification (*10*), clinical subtypes of primary lesions were as follows: 43.8% (n = 7) of mucosal melanoma, 25% (n = 4) of acral melanoma, 18.8% (n = 3) of melanoma arising from non-chronically sun-damaged (non-CSD), 6.3% (n = 1) of melanoma arising from chronically sun-damaged (CSD), and 6.3% (n = 1) of unknown. Metastasis was found in lymph node (n = 10, 62.5%), lung (n = 5, 31.3%), skin (n = 3, 18.8%), mesenterium (n = 3, 18.8%), liver (n = 2, 12.5%), digestive tract (n = 2, 12.5%), or other sites (bone, brain, kidney and adrenal glands). As prior treatment, primary lesions were surgically removed in 81.3% patients (n = 13). Additional treatment, including dacarbazine chemotherapy (n = 7, 43.8%), interferon (IFN)-β local injection (n = 2, 12.5%), and radiotherapy, (n = 2, 12.5%) was performed in 50% (n = 8) of patients.

Table 1. Clinical features of 16 melanoma patients treated with nivolumab

Factor/Category	*n* (%)
Age	
< 65	5 (31.3)
≥ 65	11 (68.8)
Median age (range)	71.3 (48-87)
Gender	
Male	7 (43.8)
Female	9 (56.3)
Stage	
III	7 (43.8)
IV	9 (56.3)
ECOG performance status	
0	14 (87.5)
1	1 (6.3)
2	1 (6.3)
3	0 (0)
4	0 (0)
Primary lesion	
Mucosal	7 (43.8)
Acral	4 (25.0)
non-CSD	3 (18.8)
CSD	1 (6.3)
Unknown	1 (6.3)
Prior therapy	
Surgery	13 (81.3)
Chemotherapy	7 (43.8)
Adjuvant IFN-β (local injection)	2 (12.5)
Radiotherapy	2 (12.5)

ECOG: Eastern Cooperative Oncology Group, Non-CSD: melanoma arising from non-chronically sun-damaged, CSD: melanoma arising from chronically sun-damaged, IFN-β: interferon-β.

2.2. *Clinical assessment*

Response assessment based on response evaluation criteria in solid tumor (RECIST) v1.1 was performed, and patients were classified into responder groups (Partial Response: PR) + Stable Disease: SD) or non-responder groups (Progressive Disease: PD). Laboratory data in this study was obtained before nivolumab treatment and approximately one month before first imaging assessment (average 77.4 days after first administration of nivolumab). Examined were: white blood cell count (WBC), by electric resistance method), absolute lymphocyte counts (ALC, by flow cytometry method), absolute neutrophil count (ANC, by flow cytometry method), absolute monocyte count (AMC, by flow cytometry method), absolute eosinophil count (AEC, by flow cytometry method), and absolute basophil count (ABC, by flow cytometry method), as well as levels of lactate dehydrogenase (LDH, by JSCC standardization compliance law), C-reactive protein (CRP, by latex immunization turbidity method), one hour value of erythrocyte sedimentation rate (ESR, by light beam detection method), and 5-S-cysteinydopa (5-S-CD, by HPLC method). This study protocol was approved by the Wakayama Medical University Ethical Committee.

2.3. *Statistical analysis*

Statistics were calculated using single-factor analysis of variance (ANOVA) and repeated measure ANOVA for comparison of median. $p < 0.05$ was considered statistically significant. Receiver operating characteristic (ROC) curve analysis was also performed using GraphPad Prism software®.

3. Results

3.1. *Side effects and clinical responses*

Immunology-related adverse events that occurred in our patients were as follows: vitiligo (n = 3), psoriaform rash (n = 2), interstitial pneumonia (n = 1, grade 3), hypothyroidism (n = 1, grade 2), thrombocytopenia (n = 1, grade 3), stomatitis (n = 1, grade 3), and nausea (n = 1, grade 3). Laboratory data abnormalities including liver enzymes and renal function were also found, although they were all grade 1 and asymptomatic. In three patients, nivolumab administration was discontinued after experience of grade 3 interstitial pneumonia, grade 2 hypothyroidism, or grade 3 nausea.

The best response rates were PD in nine patients (56.3%), SD in five patients (31.3%) and PR in two patients (12.5%). Most patients showed no change in the response after the first evaluation, except for one patient whose response changed from SD into PD after about one year.

3.2. *Baseline levels of candidates for biomarkers*

First, we examined whether baseline laboratory data before treatment can predict response to nivolumab. Blood counts, LDH, CRP, ESR, and 5-S-CD were regarded as candidates. Patients were classified into responder groups (PR+SD) and non-responder groups (PD), according to RECIST v1.1. In the responder

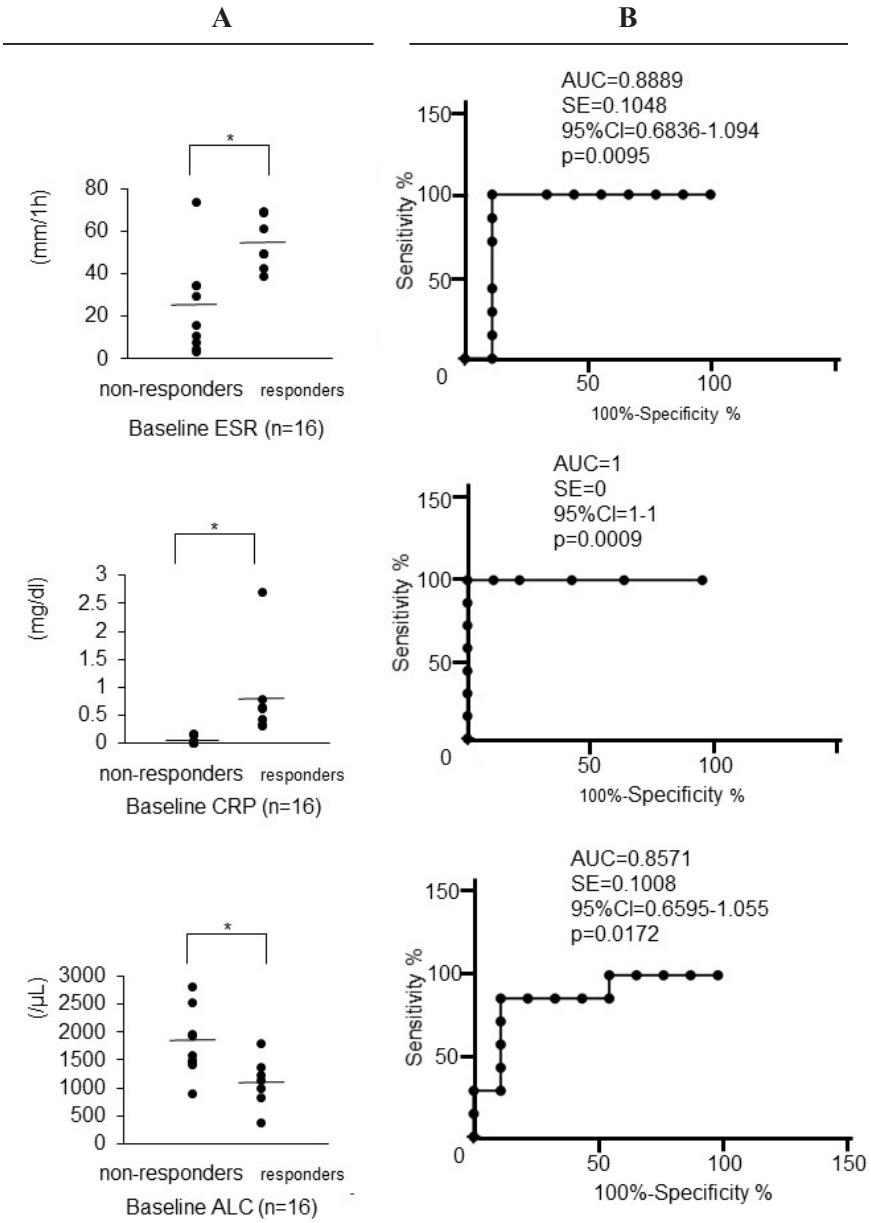

Figure 1. Baseline levels of candidates for biomarkers. (A) Baseline levels of ESR, CRP, and ALC were compared between responders (PR+SD) and non-responders (PD). Bars show means. *$p < 0.05$. **(B)** Receiver operating characteristic (ROC) curve for each laboratory data to distinguish responders from non-responders. AUC: areas under curves, SE: standard error, CI: confidence interval.

Table 2. Baseline levels of candidates for biomarkers in responders (PR+SD) and non-responders (PD)

Items	responders (n = 7)	non-responders (n = 9)	p
WBC [/μL]	5,620	5,190	0.552
ALC [/μL]	1,110	1,800	0.023*
ANC [/μL]	3,930	2,940	0.098
AMC [/μL]	359	312	0.444
AEC [/μL]	158	114	0.727
ABC [/μL]	22.9	24.4	0.932
LDH [IU/L]	211	196	0.595
CRP [mg/dL]	0.835	0.058	0.014*
ESR [mm/1h]	54.7	24.2	0.006*
5-S-CD [nmol/L]	11.0	17.2	0.213

WBC: white blood cell, ALC: absolute lymphocyte counts, ANC: absolute neutrophil count, AMC: absolute monocyte count, AEC: absolute eosinophil count, ABC: absolute basophil count, LDH: lactate dehydrogenase, CRP: C-reactive protein, ESR: erythrocyte sedimentation rate one hour value, 5-S-CD: 5-S-cysteinydopa. Mean values of each laboratory data are shown. *$p < 0.05$.

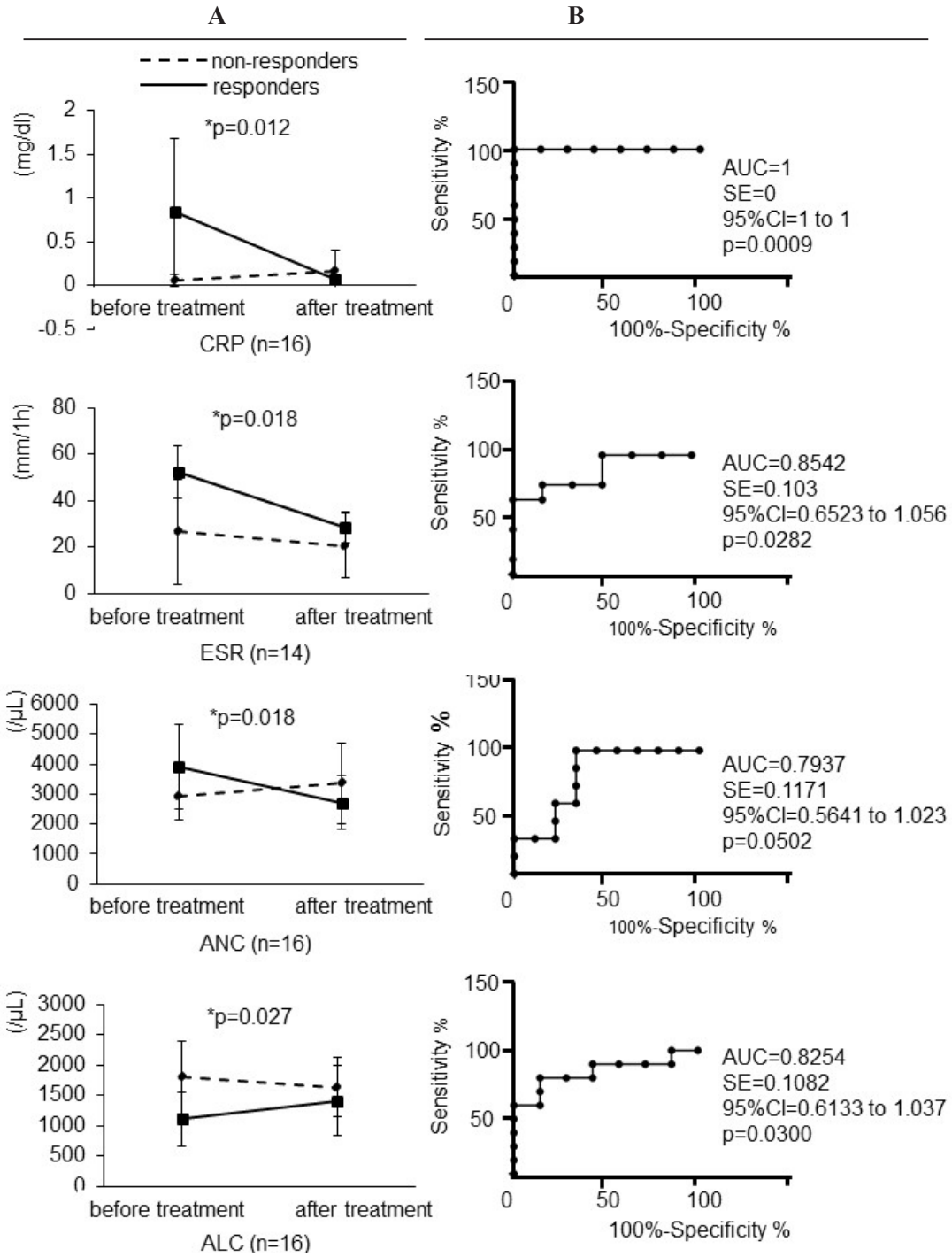

Figure 2. Changes of candidates for biomarkers before and during nivolumab treatment. (A) Graphs show mean ± standard deviation. *$p < 0.05$. **(B)** Receiver operating characteristic (ROC) curve for each laboratory data to distinguish responders (PR+SD) from non-responders (PD). AUC: areas under curves, SE: standard error, CI: confidence interval.

group, we found significantly higher ESR level (p = 0.006), higher CRP level (p = 0.014), and lower ALC level (p = 0.023), compared with non-responder group (Figure 1, left). There were no other significant differences between these groups (Table 2).

We then performed ROC curve analysis to compare the clinical usefulness of above baseline biomarkers for nivolumab treatment (Figure 1, right). Areas under curve (AUC) of baseline ESR, CRP, or ALC were 0.89 (95% CI, 0.68 to 1.09), 1.00 (95% CI, 1.00 to 1.00) or 0.86 (95% CI, 0.66 to 1.06), respectively. AUC of baseline CRP level was higher than those of other markers.

3.3. *Changes of levels of biomarker candidates in the responder group during treatment*

Increase or decrease of candidate levels after nivolumab treatment as novel biomarkers for response rate prediction were then evaluated. Laboratory data was obtained approximately one month before the first imaging assessments. In the responder group (PR+SD), there were significant differences in the levels of CRP, ESR, ANC, and ALC; ALC levels were significantly increased, while other levels were decreased after nivolumab treatment compared with the baseline (p = 0.027, 0.012, 0,018, and 0.018, respectively) (Figure 2, left). Changes of other laboratory data are shown in Table 3.

In ROC curve analysis, AUC of CRP, ESR, ANC, or ALC was 1.00 (95% CI, 1.00 to 1.00), 0.85 (95% CI, 0.65 to 1.06), 0.79 (95% CI, 0.56 to 1.02), or 0.83 (95% CI, 0.61 to 1.04), respectively (Figure 2, right). Accordingly, AUC for CRP level was higher than those for the other biomarkers.

3. 4. *Case presentation of a representative patient*

In a patient with typical PR course, baseline ESR levels and CRP levels were elevated, and ALC level was reduced before nivolumab treatment (Figure 3A). After

Table 3. Changes of values of candidates for biomarkers in responders (PR+SD) and non-responders (PD)

Items	responders		non-responders		p
	before treatment	during treatment	before treatment	during treatment	
WBC [/μL]	5,620 (n = 7)	4,680	5,190 (n = 9)	5,570	0.105
ALC [/μL]	1,110 (n = 7)	1,410	1,800 (n = 9)	1,640	0.027*
ANC [/μL]	3,930 (n = 7)	2,720	2,940 (n = 9)	3,350	0.018*
AMC [/μL]	359 (n = 7)	319	312 (n = 9)	333	0.421
AEC [/μL]	158 (n = 7)	169	114 (n = 9)	222	0.242
ABC [/μL]	22.9 (n = 7)	61.4	24.4 (n = 9)	25.6	0.123
LDH [IU/L]	211 (n = 7)	181	197 (n = 9)	211	0.073
CRP [mg/dL]	0.835 (n = 7)	0.07	0.058 (n = 9)	0.16	0.012*
ESR [mm/1h]	52.2 (n = 6)	28.5	26.6 (n = 8)	20.6	0.018*
5-S-CD [nmol/L]]	17.5 (n = 2)	6.85	14.7 (n = 5)	24.8	0.080

WBC: white blood cell, ALC: absolute lymphocyte counts, ANC: absolute neutrophil count, AMC: absolute monocyte count, AEC: absolute eosinophil count, ABC: absolute basophil count, LDH: lactate dehydrogenase, CRP: C-reactive protein, ESR: erythrocyte sedimentation rate (one hour value), 5-S-CD: 5-S-cysteinydopa. Mean values of each laboratory data are shown. *p < 0.05.

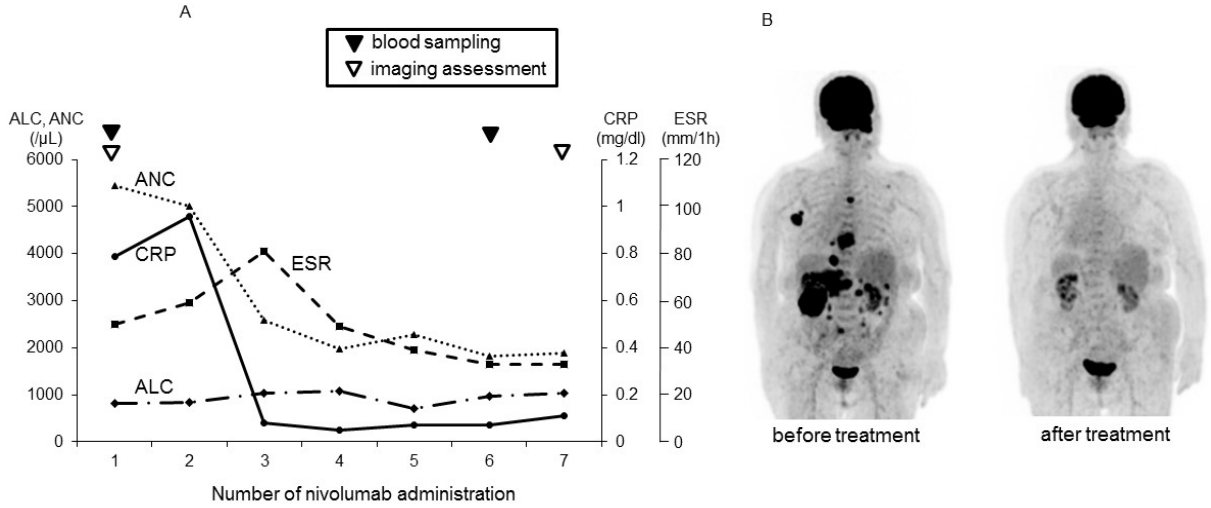

Figure 3. Case presentation of a patient with typical PR course. (A) ESR, CRP, ANC, and ALC levels before and during nivolumab treatment. Black arrowheads indicate the timing of blood sampling, and while arrowheads indicate the timing of imaging assessment. **(B)** PET imaging before and during treatment. After seven nivolumab treatments, the primary tumor of left buccal mucosa and metastatic lesions showed almost complete remission.

five courses of nivolumab, levels of CRP, ESR, and ANC were decreased, and ALC level was increased from the baseline. Positron emission tomography (PET) imaging showed the patient's tumor was markedly diminished by nivolumab (Figure 3B).

4. Discussion

Predictive biomarkers for the efficacy of nivolumab treatment against malignant melanoma have been investigated vigorously in recent years. For example, soluble CD73 levels (*11*) or Th9 cell number (*12*) in peripheral blood, expression levels of PD-L1 (*13*) or MHC-II (*14*) in tumor cells, as well as number of CD8 positive T cells (*15*) or specific inflammation and IFN-γ related mRNA-based signatures in infiltrating immune cells have been reported as clinically useful biomarkers. Furthermore, Nakamura *et al*. described serum LDH and CRP levels to be baseline prognostic markers in patients with advanced melanoma treated with nivolumab (*16*). They also indicated that patients with increased ALC and decreased ANC during nivolumab therapy had significantly better overall survival.

In this study, we determined the possibility that treatment efficacy of nivolumab can be predicted by daily blood examination. Responder group showed significantly higher baseline levels of ESR or CRP and significantly lower ALC level before the nivolumab treatment. In addition, nivolumab treatment decreased the levels of CRP, ESR, and ANC, while it increased ALC level in responder group. According to the ROC curve, CRP was most effective of these to distinguish responder group from non-responder group both before and during treatment. Patients with elevated baseline CRP levels before treatment will respond to nivolumab, and normalization of the elevated CRP by nivolumab indicates that the patients are the responder before the first imaging assessment.

Taken together, we firstly reported ESR is also the baseline biomarker of the efficacy of nivolumab. We confirm that CRP is also useful to predict efficacy both before and during treatment. CRP is suggested to be the most effective biomarker in daily blood examination using ROC curve analysis. There is a possibility that inflammation responses, reflected by elevated CRP and ESR, may be activated in the responder group before nivolumab treatment, and they are normalized during the treatment. Nivolumab treatment may therefore be more effective for malignant melanoma with strong inflammation.

Link between inflammation and cancer has been reported for over 150 years. Virchow firstly reported the association by observing white blood cells around the cancer cells in 1881 (*17*). Increased CRP has been reported to be an independent prognostic marker for malignant melanoma (*18*), correlating with the number of lymph node metastases (*19*). Indication of the association of elevated ESR with short survival has also been reported (*20*). PD-1 is expressed on effector T cells only after an immune response, such as infection or inflammation (*15*). Especially, its expression is especially remarkable on effector T cells invading the peripheral inflamed tissues. The extent of the immune response against malignant tumors usually varies depending on general condition of the patients or type of malignant tumors. Considering the inflammatory markers (CRP and ESR) in the responder group were higher before treatment, these patients have more baseline PD-1 expression due to strong immune response. Our study also suggests the synergistic effect of nivolumab therapy combined with existing treatments that causes inflammation (such as IFN-β local injection or radiation therapy).

Our study also showed increased ALC and decreased ANC levels during nivolumab therapy correlated with the better response. Neutrophil-lymphocyte ratio (NLR) is a known marker of systemic host inflammation, and it is reported that the increased NLR is associated with poor prognosis in several malignant tumors, including malignant melanoma (*21,22*), consistent with the previous paper and current study. On the other hand, lower baseline ALC before the treatment in the responder group, which was found in this study, is contradictory to this hypothesis, and may be because of the small number of patients. As the limitation, this study is retrospective study of only 16 patients. For earlier and more efficient prediction of the efficacy, the clinical significance of the combination of levels of CRP/ESR and other inflammatory markers should be evaluated in larger number of patients.

References

1. Garbe C, Eigenhler TK, Keiljolz U, Hauschild A, Kirkwood JM. Systematic review of medical treatment in melanoma: Current status and future prospects. Oncologist. 2011; 16:15-24.
2. Freeman GJ, Long AJ, Iwai Y, *et al*. Engagement of the PD-1 immunoinhibitory receptor by a novel B7 family member leads to negative regulation of lymphocyte activation. J Exp Med. 2000; 192:1027-1034.
3. Seidel JA, Otsuka A, Kabashima K. Treating tumors with immune checkpoint inhibitors: Rationale and limitations. Trends in Immunotherapy. 2017; 1:2-9.
4. Kazandjian D, Suzman DL, Blumenthal G, Mushti S, He K, Libeg M, Keegan P, Pazdur R. FDA approval summary: Nivolumab for the treatment of metastatic non-small cell lung cancer with progression on or after platinum-based chemotherapy. Oncologist. 2016; 21:634-642.
5. Borghaei H, Paz-Ares L, Horn L, *et al*. Nivolumab versus docetaxel in advanced nonsquamous non-small-cell lung cancer. N Engl J Med. 2015; 373:1627-1639.
6. Motzer RJ, Escudier B, McDermott DF, *et al*. Nivolumab versus everolimus in advanced renal-cell carcinoma. N Engl J Med. 2015; 373:1803-1813.
7. Younes A, Santoro A, Shipp M, *et al*. Nivolumab for

classical Hodgkin's lymphoma after failure of both autologous stem-cell transplantation and brentuximab vedotin: A multicentre, multicohort, single-arm phase 2 trial. Lancet Oncol. 2016; 17:1283-1294.
8. Ferris RL, Blumenschein G Jr, Fayette J, *et al*. Nivolumab for recurrent squamous-cell carcinoma of the head and neck. N Engl J Med. 2016; 375:1856-1867.
9. Larkin J, Chiarion-Sileni V, Gonzalez R, *et al*. Combined nivolumab and ipilimumab or monotherapy in untreated melanoma. N Engl J Med 2015; 373:23–34.
10. Curtin JA, Fridlyand J, Kageshita T, Patel HN, Busam KJ, Kutzner H, Cho KH, Aiba S, Bröcker EB, LeBoit PE, Pinkel D, Bastian BC. Distinct sets of genetic alterations in melanoma. N Engl J Med. 2005; 353: 2135-2147.
11. Morello S, Capone M, Sorrentino C, Giannarelli D, Madonna G, Mallardo D, Grimaldi AM, Pinto A, Ascierto PA. Soluble CD73 as biomarker in patients with metastatic melanoma patients treated with nivolumab. J Transl Med. 2017; 15:244.
12. Nonomura Y, Otuka A, Nakashima C, *et al*. Peripheral blood Th9 cells are a possible pharmacodynamic biomarker of nivolumab treatment efficacy in metastatic melanoma patients. Oncoimmunology. 2016; 5:e1248327.
13. Gandini S, Massi D, Mandala M. PD-L1 expression in cancer patients receiving anti PD-1/PD-L1 antibodies: A systematic review and meta-analysis. Crit Rev Oncol Hematol. 2016; 100:88-98.
14. Johnson DB, Estrada MV, Salgado R, *et al*. Melanoma-specific MHC-II expression represents a tumour-autonomous phenotype and predicts response to anti-PD-1/PD-L1 therapy. Nat Commun. 2016; 7:10582.
15. Erdag G, Schaefer JT, Smolkin ME, Deacon DH, Shea SM, Dengel LT, Patterson JW, Slingluff CL Jr. Immunotype and immunohistologic characteristics of tumor-infiltrating immune cells are associated with clinical outcome in metastatic melanoma. Cancer Res. 2012; 72:1070-1080.
16. Nakamura Y, Kitano S, Takahashi A, Tsutsumida A, Namikawa K, Tanese K, Abe T, Funakoshi T, Yamamoto N, Amagai M, Yamazaki N. Nivolumab for advanced melanoma: Pretreatment prognostic factors and early outcome markers during therapy. Oncotarget. 2016; 7:77404-77415.
17. Virchow R. An address on the value of pathological experiments. Br Med J. 1881; 2:198-203.
18. Fang S, Wang Y, Sui D, *et al*. C-reactive protein as a marker of melanoma progression. J Clin Oncol. 2015; 33:1389-1396.
19. Neuss H, Koplin G, Raue W, Reetz C, Mall JW. Analysing the serum levels of tumour markers and primary tumour data in stage III melanoma patients in correlation to the extent of lymph node metastases – a prospective study in 231 patients. Acta Chir Belg. 2011; 111:214-218.
20. Tas F, Erturk K. Elevated erythrocyte sedimentation rate is associated with metastatic disease and worse survival in patients with cutaneous malignant melanoma. Mol Clin Oncol. 2017; 7:1142-1146.
21. Lino-Silva LS, Salcedo-Hernández RA, García-Pérez L, Meneses-García A, Zepeda-Najar C. Basal neutrophil-to-lymphocyte ratio is associated with overall survival in melanoma. Melanoma Res. 2017; 27:140-144.
22. Templeton AJ, Knox JJ, Lin X, *et al*. Change in neutrophil-to-lymphocyte ratio in response to targeted therapy for metastatic renal cell carcinoma as a prognosticator and biomarker of efficacy. Eur Urol. 2016; 70:358-364.

(Received July 9, 2018; Revised August 14, 2018; Accepted August 17, 2018)