Original Article

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Increase of lymphocytes and eosinophils, and decrease of neutrophils at an early stage of anti-PD-1 antibody treatment is a favorable sign for advanced malignant melanoma

Hiroyuki Ohashi, Sora Takeuchi, Tomomitsu Miyagaki, Takafumi Kadono*

Department of Dermatology, St. Marianna University School of Medicine, Kawasaki, Kanagawa, Japan.

SUMMARY The advent of immune checkpoint inhibitors such as anti-PD-1 antibodies had a striking impact on the treatment for advanced malignant melanoma. However, less than half of the patients benefited from those antibodies, and biomarkers that could sensitively differentiate responders from non-responders are urgently needed. Herein, we explored such biomarkers by retrospectively analyzing clinical data from patients with advanced malignant melanoma treated with nivolumab and pembrolizumab. We found that anti-PD-1 antibody was especially effective for those with metastasis only to soft tissues. Although no significant difference was found in the baseline value of relative neutrophil count (RNC), relative lymphocyte count (RLC), neutrophil to lymphocyte ratio (NLR), and relative eosinophil count (REC) between responders and non-responders, responders after anti-PD-1 therapy revealed the increase of lymphocytes and eosinophils and the decrease of neutrophils within the first 6 weeks of the treatment. We also calculated the change of RNC and RLC 3 weeks and 6 weeks after the initiation of the therapy and designated as NΔ3-LΔ3 and NΔ6-LΔ6 respectively. NΔ3-LΔ3 was significantly decreased in responders, which suggest that the neutrophil decrease and lymphocyte increase after as early as 3 weeks of anti-PD-1 therapy might be a useful clinical indicator. In addition, the difference of NΔ6-LΔ6 between responders and non-responders was even more robust. These data suggest that change of RNC, RLC, and REC together with the combination of NΔ3-LΔ3 and NΔ6-LΔ6 might be a useful tool for early and sensitive biomarkers for anti-PD-1 therapy.

Keywords malignant melanoma, nivolumab, pembrolizumab, relative neutrophil count, relative lymphocyte count, biomarker

1. Introduction

The introduction of anti-PD-1 antibody has dramatically changed the treatment for advanced malignant melanoma. Since the advent of nivolumab in July 2014, nivolumab and pembrolizumab are widely used worldwide for unresectable or metastatic melanomas. Although the clinical effects of anti-PD-1 antibodies are unprecedented, anti-PD-1 antibodies are effective only in about 30% of Japanese patients with advanced malignant melanoma (*1*). Under the present circumstances, there is only limited information regarding which type of patients this treatment protocol is more beneficial. Biomarkers for predicting the therapeutic effects of anti-PD-1 antibodies are still obscure, and being actively studied throughout the world.

Sex might affect the therapeutic effect, and men have longer overall survival (OS) and progression free survival (PFS) than women (*2*). In contrast, age is not known to affect the prognosis. Immune checkpoint inhibitors (ICI) have been reported to prolong OS in both young and elderly patients (cut-off 65-70 years), and no significant difference in the average age was found between responders and non-responderrs (*3*). Elevated lactate dehydrogenase (LDH), elevated C-reactive protein (CRP), and Eastern Cooperative Oncology Group performance status (ECOG PS) ≥ 1 have been reported to shorten OS (*4*), and patients with no metastases other than soft tissue and lung have been reported to fare well (*5*).

One of the promising biomarkers is blood cell count. For example relative eosinophil count (REC) ≥ 1.5 was reported to be associated with good OS (*5*). In addition, reduction of neutrophil to lymphocyte ratio (NLR) 6 weeks after the first dose of anti-PD-1 antibody suggests better responses (*6*), and an increase of 30% or more in NLR within the first two cycles of anti-PD-1 antibody was associated with worsening of OS and reduction of

time to treatment failure (*7*).

To elucidate the factors and biomarkers that could sensitively discriminate responders and non-responders, we conducted a retrospective single-center study of patients with unresectable malignant melanoma.

2. Materials and Methods

2.1. Patient

Sixteen patients with unresectable malignant melanoma (stage III-IV) who received nivolumab or pembrolizumab treatment from February 2015 to March 2020 were enrolled in this study. Twelve patients received nivolumab and 4 patients received pembrolizumab. A patient who discontinued treatment after the first dose and was transferred to another hospital was excluded. Relevant prognostic factors that included age, gender, stage, ECOG PS, presence or absence of BRAF mutation, metastatic site, LDH, CRP, RLC, RNC, NLR, and REC were analyzed to measure the treatment response among those patients. Within 21 days prior to anti-PD-1 antibody administration, patients underwent a blood test as a baseline. LDH, CRP, RNC, RLC, NLR, and REC were measured at 3 weeks and 6 weeks after initiation of treatment, and their time-courses were monitored. Five patients had a blood draw at week 4 instead of week 3. Three patients had blood draws at 7 or 8 weeks instead of week 6. This study has been approved by the Research Ethics Committee of St. Marianna University School of Medicine.

2.2. Treatment and response

Nivolumab was started at 2 mg/kg every 3 weeks in 9 patients. Three of them was later changed to 240 mg/body every 2 weeks. In the other 3 patients, the dose started at 240 mg/body every 2 weeks. Pembrolizumab was started at 2 mg/kg every 3 weeks in 4 patients, and one of them was later changed to 200 mg/body every 3 weeks. Patients were classified as responders (complete response: CR + partial response: PR) or non-responders (stable disease: SD + progressive disease: PD) using Response Evaluation Criteria in Solid Tumors (RECIST) version 1.1 at 6 months after anti-PD-1 antibody administration.

2.3. Statistical analysis

Fisher's exact test was used to compare the ratio of nominal variables between two independent groups. Repeated measures analysis of variance was used to compare continuous variables before, 3 weeks after, and 6 weeks after administration. A *t*-test was used to compare continuous variables before and after administration. A *p*-value of 0.05 or less was considered statistically significant. All data was analyzed using EZR (*8*).

3. Results

3.1. Patient characteristics

The characteristics of the 16 patients are shown in Table 1. The average age was 74.6 years (range 51-88 years), with 7 (43.8%) patients aged 75 years or older and 9 (56.3%) younger than 75 years. The gender was 8 males (50%) and 8 females (50%). The stage was 8 in stage 3 (50%) and 8 in stage 4 (50%). The ECOG PS was 11 in PS 0 (68.8%), 4 in PS 1 (25%), and 1 in PS 2 (6.3%). Three (18.8%) had BRAF mutations and 13 (68.8%) did not. Patients with low-cumulative sun damage (CSD) were 6 cases (37.5%), high-CSD were 2 cases (12.5%), and 7 patients were low to no-CSD, of which 4 were acral (25%) and 3 were mucosal (18.8%). The primary site was unknown in 1 patient (6.3%). Ten (62.5%) patients had metastasis of lymph nodes/soft tissue only, while 6 (37.5%) had metastasis to other organs. Nine (56.3%) patients had received prior therapy, including 6 in adjuvant interferon-β (IFN-β) (37.5%), 2 in adjuvant DAV-Feron (dacarbazine, nimustine, vincristine, and interferon-β) (12.5%), and 3 in dabrafenib + trametinib (18.8%), and 1 patient (6.3%) had dacarbazine (including duplicates). Baseline LDH ranged from 121 to 886 (U/L). Five patients (31.3%) exceeded the reference value (115-230), and 11 patients (68.8%) were below the reference value. Baseline CRP ranged from less than 0.03 to 1.32 (mg/dL). Four patients (25%) exceeded the reference value (< 0.3), and 12 (75%) were below the reference value (Table 1).

Table 1. Patient demographics

Factor	Category	*n* (%)
Age	< 75	7 (43.8)
	> 75	9 (56.3)
Gender	Male	8 (50.0)
	Female	8 (50.0)
Stage	III	8 (50.0)
	IV	8 (50.0)
ECOG PS	0	11 (68.8)
	1	4 (25.0)
	2	1 (6.3)
BRAF mutation	presence	3 (18.8)
	absence	13 (68.8)
Primary site	Low-CSD	6 (37.5)
	High-CSD	2 (12.5)
	Low to no-CSD	
	acral	4 (25)
	mucosal	3 (18.8)
	Unknown	1 (6.3)
Site of metastasis	only lymph node and soft tissue	10 (62.5)
	other	6 (37.5)
Prior therapy	adjuvant IFN-β	6 (37.5)
	adjuvant DAV-Feron	2 (12.5)
	Dabrafenib + Trametinib	3 (18.8)
	Dacarbazine	1 (6.3)
Baseline LDH	< 230	5 (31.3)
	> 230	11 (68.8)
Baseline CRP	< 0.3	4 (25)
	> 0.3	12 (75)

3.2. Immune-related adverse events (irAE)

IrAEs of Grade 2 or higher include: hypoadrenocorticism (Grade 2) in 1 patient, hypophysitis (Grade 3) in 1, hypothyroidism (Grade 2) in 3, and type 1 diabetes in 1 (Grade 3), 1 with liver dysfunction (Grade 3), 1 with pancreatitis (Grade 2), 1 with renal dysfunction (Grade 3), and 1 with interstitial pneumonia (Grade 3). Six of the patients had vitiligo and 2 had erythema (Table 2).

3.3. Clinical responses and survival

The response to the anti-PD-1 antibody was 2 in CR, 4 in PR, 1 in SD, and 9 in PD. Six patients (37.5%) were responders (CR + PR), and 10 patients (62.5%) were non-responders (SD + PD) (Table 3).

3.4. Assessment of pretreatment prognostic factors

Patients with lymph node/soft tissue metastases responded better than patients with other organ metastases (p = 0.044). The average age of responders was 77.8 years, and the average age of non-responders was 72.7 years, with no significant difference (p = 0.342). No significant difference in treatment response was found by gender, stage, ECOG PS of 0 or more, and the presence or absence of BRAF gene mutation (p = 1, 0.119, 1, and 1, respectively). There was also no significant difference in treatment response by baseline LDH and baseline CRP (p = 0.588 and 1, respectively). No significant difference was also noted when the patients were divided by baseline RNC, RLC, NLR, and REC (p = 0.683, 0.121, 0.269, and 0.3, respectively) (Table 4).

Table 2. IrAE of Grade 2 or higher

IrAE	Grade	*n*
hypoadrenocorticism	2	1
hypophysitis	3	1
hypothyroidism	2	3
type 1 diabetes	3	1
pancreatitis	2	1
renal dysfunction	3	1
interstitial pneumonia	3	1

Table 3. Clinical responses

	n	Response	*n*
Responder	6 (37.5%)	CR	2 (12.5%)
		PR	4 (25.0%)
Non-responder	10 (62.5%)	SD	1 (6.3%)
		PD	9 (56.3%)

3.5. Changes of biomarkers through the early phase of the treatment

As responders did not show any significant differences in the baseline of various biomarkers, we next examined the sequential changes of these biomarkers before, 3 weeks after, and 6 weeks after anti-PD-1 therapy. Notably, RNC was significantly decreased and RLC was significantly increased in responders compared to non-respoders after 6 weeks (p = 0.024, 0.00038, respectively). Reflecting the increase of RLC and the decrease of RNC, NLR was significantly decreased in responders (p = 0.018) (Figure 1). However, no significant difference was found in LDH and CRP (p = 0.382, 0.265, respectively).

3.6. Difference of NΔ3-LΔ3 and NΔ6-LΔ6 between responders and non-responders

As for RLC and RNC, we noticed the difference between responders and non-responders steadily widened from baseline to week 3, and then to week 6. Therefore, we calculated the change of RNC and RLC values to find more sensitive early biomarkers that could differentiate responders from non-respomders. We designated the value of RNC at week 3 minus its baseline value as NΔ3, and the value of RLC at week 3 minus its baseline value as LΔ3. In a similar manner,

Table 4. Assessment of prognostic factors and early biomarkers before the therapy

Factor	Responder (n = 6)	Non-responder (n = 10)	*P*
Lymph node/soft tissue metastases only[*1]	6	4	0.044
Age	77.8 (64-88)	72.7 (51-83)	0.342
Gender (male)	3	5	1
Stage (IV)	1	7	0.119
ECOG PS (> 0)	2	3	1
BRAF mutation	1	2	1
Baseline LDH (> 230)	1	4	0.588
Baseline CRP (> 0.3)	2	3	1
Baseline RNC[*2]	63.0 (40.5-72.2)	61.1 (49.5-69.1)	0.683
Baseline RLC[*2]	23.7 (19.3-28.0)	27.2 (20.7-35.2)	0.121
Baseline NLR[*2]	2.7 (1.6-3.7)	2.3 (1.4-3.3)	0.269
Baseline REC[*2]	1.77 (0.3-3.4)	1.03 (0.5-2.08)	0.3
REC > 1.75 (6 weeks after administration)[*2]	3	0	0.044

[*1]One case with only local recurrence is not included. [*2]One data is missing.

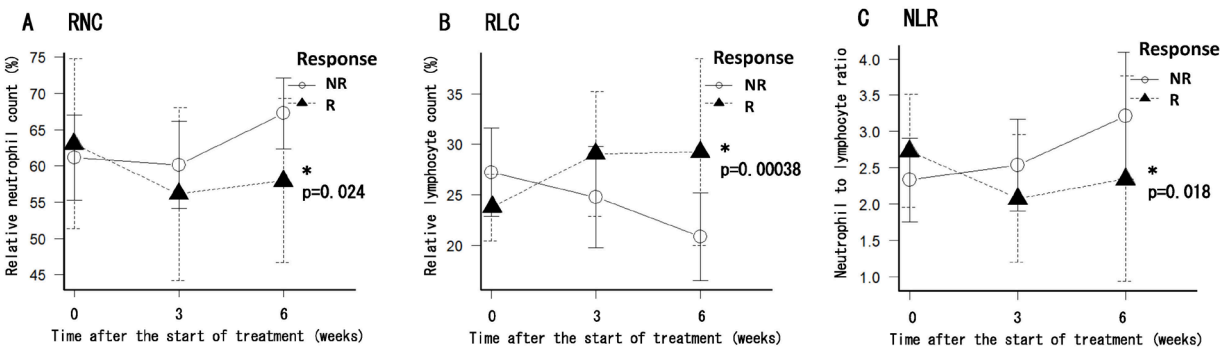

Figure 1. Time course of RNC, RLC, and NLR at 0, 3, and 6 weeks after anti-PD-1 antibody treatment. (A) RNC decreased in responders, and increased in non-responders. The difference between responders, and non-responders was significant at week 6 (p = 0.024). **(B)** RLC increased in responders, whereas it decreased for non-responders. The difference between responders, and non-responders was significant at week 6 (p = 0.00038). **(C)** NLR was significantly decreased in responders (p = 0.018).

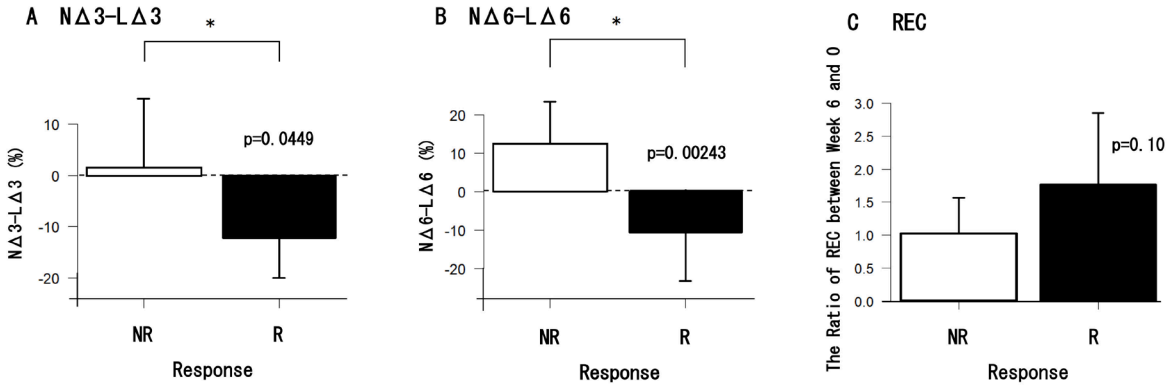

Figure 2. Difference of NΔ3-LΔ3 and NΔ6-LΔ6 between responders and non-responders. (A) The value of NΔ3-LΔ3 at week3 was decreased in responders. The difference between responders, and non-responders was significant (p = 0.044). **(B)** The value of NΔ3-LΔ3 at week6 was decreased in responders and increased in non-responders. The difference between responders, and non-responders was significant (p = 0.00234). **(C)** The ratio of REC between week 6 and its baseline tended to be increased in responders compared to non-responders (p = 0.10).

NΔ6 was designated as the value of RNC at week 6 minus its baseline, and LΔ6 as the RLC at week 6 minus its baseline. We found that NΔ3-LΔ3 was significantly decreased in responders (-12.3%; range from -1.7 to -20.5) compared to non-responders (1.4%; range from -8.9 to 35.1) (p = 0.0449). The change of NΔ6-LΔ6 was even more striking, with -10.6% in responders compared to +12.4% in non-responders (Responders range from 5.5 to -21.8. Non-responders range from -4.4 to 27.9. p = 0.00243) (Figure 2).

Of note, while the change between NΔ3-LΔ3 and NΔ6-LΔ6 was subtle in responders, NΔ6-LΔ6 (+12.4%) was substantially increased compared to NΔ3-LΔ3 (+1.4%) in non-responders. In addition, the ratio of REC between week 6 and its baseline tended to be increased in responders compared to non-responders (p = 0.10). In 3/6 of responders, the value of REC at 6 weeks was more than1.75 times of baseline REC, whereas there were none (0/10) in non-responders (p = 0.044) (Table 4).

These data demonstrate that within 6 weeks of anti-PD-1 treatment, neutrophils tend to decrease and lymphocytes tend to increase in responders, and non-responders show the opposite tendency.

4. Discussion

In the current study, we found that responders after anti-PD-1 therapy revealed the increase of lymphocytes and eosinophils and the decrease of neutrophils within the first 6 weeks of the treatment. We also generated more sensitive clinical indicators, NΔ3-LΔ3 and NΔ6-LΔ6, that discriminate between responders and non-responders. NΔ3-LΔ3 was significantly decreased in responders, which suggest that the neutrophil decrease and lymphocyte increase after as early as 3weeks of anti-PD-1 therapy might be a useful clinical indicator. In addition, the difference of NΔ6-LΔ6 between responders and non-responders was even more robust. These data suggest that the combination of NΔ3-LΔ3 and NΔ6-LΔ6 might be a useful tool for early (NΔ3-LΔ3) and sensitive (NΔ6-LΔ6) dection of anti-PD-1 efficacy.

Several predictive factors for better ICI responses have recently been reported, such as sex, age,

performance status, and site of metastasis. In our study, no significant difference was found in terms of age, sex, and performance status between responders and non-responders. As for the site of metastasis, metastasis to soft tissue alone was associated with good response as previously reported (*5*). Therefore, it is preferable that ICI could be administered before metastasizing to distant organs.

LDH and CRP has been reported as useful biomarkers (*4*). However, we could not find significant difference between responders and non-responders. Instead, we could confirm the usefulness of REC, and the increase of REC at 6 weeks suggested the better response.

The hallmark of current studty is to show the usefulness of NΔ3-LΔ3 and NΔ6-LΔ6. Although the baseline NLR exceeding 2.2-5 indicated the sign of poor prognosis in previous studies (*6,7,9*), we could not detect significant difference regarding the baseline NLR. However, the increase of RLC, and the decrease of RNC and NLR during the course of anti-PD-1 therapy were associated with better treatment response. In previous studies, the reduction of NLR at week 6 suggested the better response (*6*), and the increse of NLR within the first two cycles of anti-PD-1 antibody indicated worsening of OS (*7*). We utilize this notion and calculated NΔ3-LΔ3 and NΔ6-LΔ6. NΔ3-LΔ3 reflects the change of neutrophils and lymphocytes at week 3 and could be used as an early biomarker, and NΔ6-LΔ6 reflects the change at week 6 and could be used as a sensitive biomarker to judge the effect. Although this formula was somewhat complicated and this retrospective study consisted of only limited number of patients, NΔ3-LΔ3 and NΔ6-LΔ6 could be useful to estimate the therapeutic effect of anti-PD-1 antibodies at an earlier stage.

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**Address correspondence to:*
Takafumi Kadono, Dermatology, St Marianna University School of Medicine, 2-16-1 Sugao, Miyamae, Kawasaki, Kanagawa 216-8511, Japan.
E-mail: tkadono@marianna-u.ac.jp

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