Potential of circulating immune cells as biomarkers of nivolumab treatment efficacy for advanced hepatocellular carcinoma

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Abstract

Background: Remarkable progress has been made in immunotherapy, specifically antibodies for programmed death 1 (PD-1) or programmed death-ligand 1 (PD-L1), for treating advanced cancers. In this study, we explored whether circulating immune cells can be used as biomarkers of the efficacy of such therapy.

Methods: We enrolled patients who received nivolumab, an anti-PD-1 antibody, for advanced hepatocellular carcinoma (HCC) in clinical trials and who consented to the collection of their peripheral blood. Using flow cytometry, we analyzed lymphocyte subclasses and the PD-1 or PD-L1 positivity of immune cells. These results were compared between patients with disease control (complete response, partial response, or stable disease) and those with disease progression.

Results: This study included 16 patients. The objective response rate was 19%, and the disease control rate was 75%. The hemogram results and the percentage of total αβ T cells or CD4 T cells did not significantly change after nivolumab treatment; moreover, they were not associated with treatment outcomes. The number of CD8 T cells significantly increased after 4 weeks ($p = 0.016$); however, this change was not associated with treatment outcomes. Patients with disease control exhibited peripheral B cells with significantly lower pretreatment PD-1 positivity than did patients with disease progression ($p = 0.042$). Patients with disease progression were more likely to exhibit monocytes with increased PD-L1 positivity after 28 ($p = 0.020$) or 42 ($p = 0.008$) days of treatment.

Conclusion: The low pretreatment PD-1 positivity of peripheral B cells and the constant posttreatment PD-L1 positivity of monocytes were associated with disease control after nivolumab treatment for advanced HCC.

Keywords: Flow cytometry; Hepatocellular carcinoma; Immunotherapy; Nivolumab; Predictive markers

1. INTRODUCTION

Treatment of advanced hepatocellular carcinoma (HCC) remains a clinical challenge. Previously, sorafenib was the only first-line approved medication for advanced HCC worldwide because it significantly increased overall survival (OS).[1,2] However, in a phase III trial,[3] lenvatinib was proven to be noninferior to sorafenib for OS. Moreover, regorafenib has been proven to provide survival benefits and can thus be used as second-line therapy in patients after failure of sorafenib treatment. However, sorafenib, lenvatinib, or regorafenib exhibit moderate efficacy.[4] The median time to progression (TTP) of sorafenib is only 2.8 months in the East Asian population. Lenvatinib is associated with a TTP of 8.9 months. Novel effective therapy for advanced HCC is a highly unmet need.

Remarkable progress has been made in immunotherapy, specifically immune checkpoint inhibitors, for treating advanced cancers. Nivolumab, which is a checkpoint inhibitor, is a human IgG4 antiprogrammed death 1 (PD-1) monoclonal antibody. When administered either in combination or alone, it has been proven to be effective for several cancer types, even those refractory to current therapy.[5–12] In CheckMate 040,[13] a multicenter, noncomparative, open-label, phase 1/2 study, El-Khoueiry et al. proved the survival benefits of nivolumab in patients with advanced HCC who received prior sorafenib therapy. In the dose-escalation phase, the objective response rate was 15%–20%, and the disease control rate was 58%, which increased to 64% in the dose-expansion phase. The rates are superior to those of current treatment options. Based on the results of that study, in September 2017, the United States Food and Drug Administration (FDA) accepted the priority review application for nivolumab in previously treated patients with advanced

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HCC. The role of immunotherapy in advanced HCC was therefore established.

Although nivolumab is a breakthrough immunotherapy for advanced HCC, its objective response rate was reported to be approximately 20%.[13] Identifying the appropriate patient group for immunotherapy is crucial. Researchers have proposed several markers of the treatment outcome. However, none of them was optimal until now. Theoretically, circulating immune cells are targets of immune checkpoint inhibitors and may be altered during treatment. The dynamic changes in immune cells may reflect the effect of immune checkpoint inhibitors and may predict the treatment outcome. According to a review of the clinical data from studies of anti-PD-1 therapy,[6–8,10–13] objective responses occurred within 3 months in most of these studies. The alteration of the immune system caused by anti-PD-1 therapy must occur earlier and should be systemic detectable. However, studies investigating the dynamic changes in peripheral immune cells after anti-PD-1 therapy are lacking.

Therefore, we conducted an observational study of the advanced HCC patient population of CheckMate 040 who were enrolled from National Taiwan University Hospital and Taipei Veterans General Hospital.[13] We hypothesized that circulating immune cells are correlated with the treatment outcomes of patients with advanced HCC; thus, we identified and mapped the dynamic changes in functional immune cells after nivolumab therapy.

2. METHODS

2.1. Study Design

This single-arm observational study aimed to determine predictive biomarkers in patients treated with the anti-PD-1 antibody nivolumab. All patients had been enrolled in the dose-expansion phase of a phase 1 clinical trial. The key eligibility criteria of the trial were histologically confirmed advanced HCC, Child-Pugh class A liver reserve, stable performance status, and adequate organ function. These patients received 3 mg/kg nivolumab through intravenous infusion every 2 weeks until they showed disease progression. Tumor assessment was performed every 6 weeks according to RECIST version 1.1.

Enrollment into this biomarker study was optional for these patients. For patients who provided consent, we collected their peripheral blood before nivolumab treatment and then 4 and 6 weeks after the start of nivolumab treatment. This biomarker study was approved by the Research Ethics Committee of National Taiwan University Hospital and Taipei Veterans General Hospital. We have confirmed that all research was performed in accordance with relevant guidelines and regulations, and the informed consent was obtained from all participants or their legal guardians.

The healthy individuals were recruited in our institute (Taipei Veterans General Hospital) and National Taiwan University Hospital. The key inclusion criteria were adults who were older than 20 years old, no major diseases such as hypertension, diabetes mellitus, no autoimmune diseases, and no malignant diseases. Informed consent was also provided with comprehensive description of the risk in this study.

2.2. Measurement of Immune Cells in Peripheral Blood

We collected peripheral blood mononuclear cells (PBMCs) through Ficoll density gradient centrifugation (Ficoll-Paque Premium; GE Healthcare). After they were resuspended with staining buffer (1× PBS with 0.5% bovine serum albumin and 0.5 mM EDTA), 2.5×10^5 PBMCs were labeled with the following cell surface markers: CD4 PE-Cy7 (SFCI12T4D11), CD8 Pacific Blue (RPA-T8), CD56 APC-Alexa Fluor 700 (N901(NKH-1)), CD3 Krome orange (UCHT1), CD14 APC-Alexa Fluor 750 (RMO52), CD19 APC-Alexa Fluor 750 (J3-119) (Beckman Coulter, Brea, CA); CD25 PE (BC96), CD11c APC (3.9), PD-1 Alexa flour 488 (EH12.2H7), programmed death-ligand 1 PE (PD-L1 PE) (29E.2A3), T cell receptor (TCR) α/β (TCR α/β FITC) (IP26), and TCR γ/δ APC (B1) (BioLegend, San Diego, CA). Some samples were further permeabilized and stained with antihuman Foxp3 APC (236A/E7) (eBioscience, San Diego, CA) and anti-CTLA-4 PE (L3D10) (BioLegend) antibodies. Data were acquired using a Navios flow cytometer (Beckman Coulter) and were analyzed using Kaluza software version 1.3 (Beckman Coulter).

2.3. Statistical Analysis

All statistical analyses were performed using SAS statistical software (version 9.1.3; SAS Institute, Cary, NC), and 2-sided $p < 0.05$ was considered significant. We used the independent t test to compare the hemogram results and specific lymphocyte subclass percentages of the study patients and healthy volunteers as well as those of patients with disease control and disease progression. Because of the small sample size and non-normal distributive population, Mann-Whitney and Wilcoxon rank test were used to compare pretreatment and posttreatment changes in parameters. The TTP was estimated using the Kaplan-Meier method.

3. RESULTS

3.1. Patient Characteristics and Treatment Outcome

This study included 16 patients; only 1 patient was female (Table 1). The median age was 62 years, and 63% of patients were seropositive for the hepatitis B virus surface antigen. All

Table 1

Patient demographics

Variables	Patient Number	%
Total	16	100
Median age (range, in y)	62 (38–75)	
Female/male	1/15	6/94
Hepatitis virus		
HBsAg positive	10	63
Anti-HCV positive	3	19
Median AFP (IQR, in mg/dL)	304.38 (10250.7075)	
Child-Pugh class		
A	16	100
BCLC stage		
C	16	100
Extrahepatic metastasis	14	88
Macroscopic vascular invasion	7	44
Previous treatment	16	100
Hepatectomy	11	68.75
Local treatment (PEIT/RFA)	8	50
TACE	10	62.5
ECOG PS		
0	2	13
1	14	88
Best objective response		
Complete response	0	0
Partial response	3	19
Stable disease	9	56
Progressive disease	4	25

AFP = α-fetoprotein; BCLC = Barcelona-clinic liver cancer; ECOG PS = Eastern Cooperative Oncology Group performance status; HBsAg = hepatitis B surface antigen; HCV = hepatitis C virus; PEIT = percutaneous ethanol injection therapy; RFA = radiofrequency ablation; TACE = transcatheter arterial chemoembolization

patients had Child-Pugh class A liver reserve and Barcelona-Clinic Liver Cancer C stage disease. None of the patients showed a complete response (CR); however, 3 (19%) patients showed partial responses (PRs) and 9 (56%) were in the stable disease (SD) stage. Other four patients had progressed disease. The overall response rate was 19%, and the disease control rate (CR + PR + SD) was 75%. The median TTP was 2.7 months.

Comparing the hemogram results before and after nivolumab treatment, no significant changes were observed in the leukocyte, neutrophil, lymphocyte, monocyte, and eosinophil counts (Supplementary Fig. 1, http://links.lww.com/JCMA/A66). The neutrophil/lymphocyte (N/L) ratio also remained constant. After both 4 and 6 weeks of nivolumab treatment, the percentage of peripheral CD4 ($p < 0.001$; Fig. 1A) and CD8 ($p < 0.001$; Fig. 1B) T cells showing PD-1 positivity significantly decreased, implying that nivolumab was bound to PD-1 receptors on these T cells.

3.2. Peripheral αβ CD8 T Cells Increased After Nivolumab Treatment

We gated PBMCs to select $CD3^+CD56^-CD14^-CD19^-$ cells and analyzed the distribution of αβ T cells and γδ T cells (Supplementary Fig. 2A and 2B, http://links.lww.com/JCMA/A67). The percentage of αβ T cells in the peripheral blood remained constant after nivolumab treatment (Fig. 1C), and

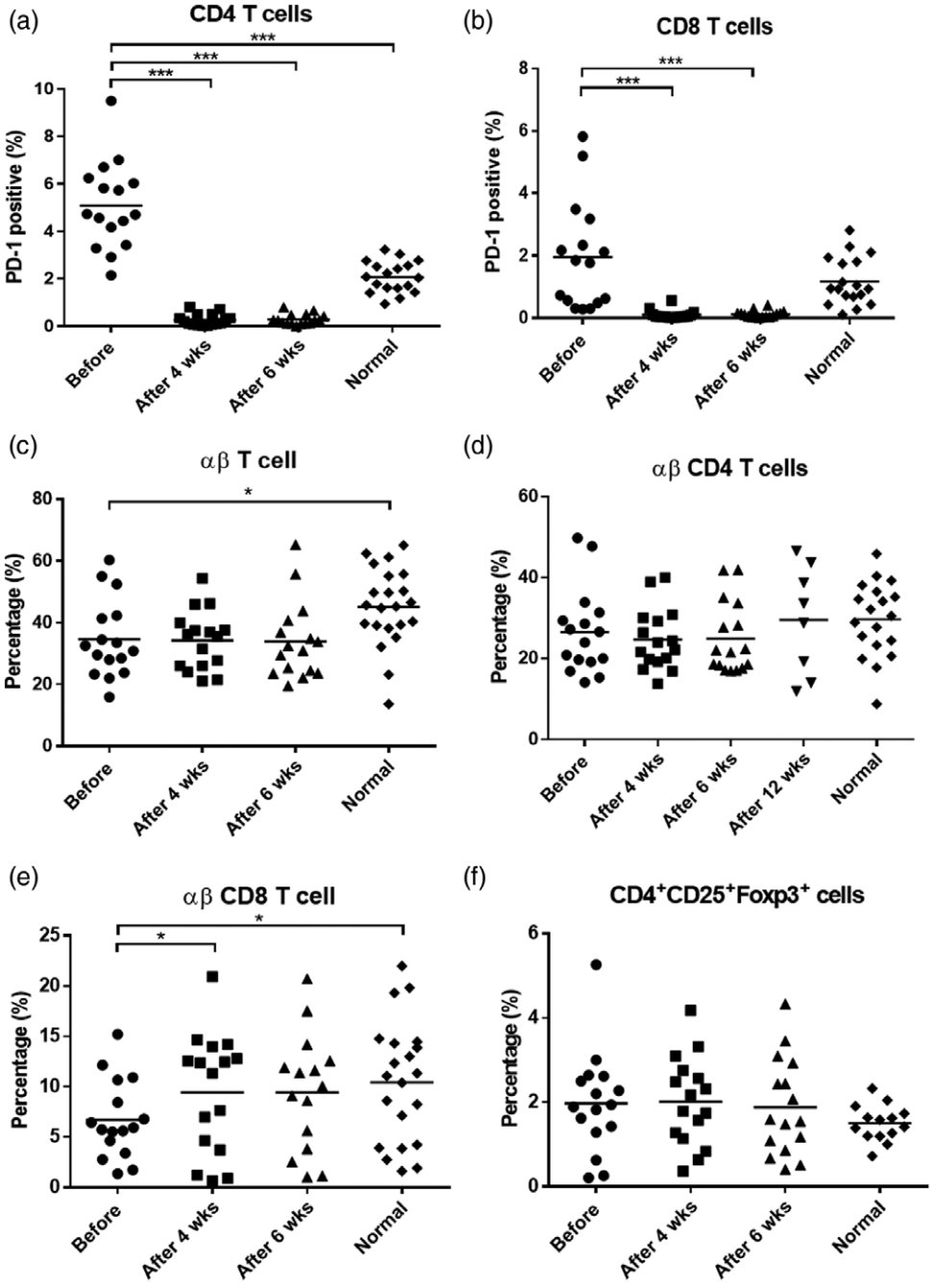

Fig. 1 (A and B) Percentage of (A) CD4 and (B) CD8 T cells with PD-1 positivity. (C–F) Pretreatment and posttreatment percentages of (C) total αβ T cells, (D) CD4 αβ T cells, (E) CD8 αβ T cells, and (F) $CD4^+CD25^+Foxp3^+$ regulatory T cells in the peripheral blood (***$p < 0.001$; *$p < 0.05$).

the percentage of CD4 αβ T cells also remained constant after nivolumab treatment (Fig. 1D). By contrast, the percentage of CD8 αβ T cells significantly increased after 4 weeks of nivolumab treatment ($p = 0.016$) and borderline significantly increased after 6 weeks of nivolumab treatment ($p = 0.064$) (Fig. 1E). In addition, we found that patients with HCC had a lower percentage of total αβ T cells and CD8 αβ T cells than did healthy volunteers (Fig. 1C and E; both $p < 0.05$). The percentage of CD4 regulatory T ($CD4^+CD25^+Foxp3^+$) cells remained constant after treatment (Supplementary Fig. 2C, http://links.lww.com/JCMA/A67; Fig. 1F).

3.3. Peripheral Immune Cells and Treatment Outcomes

Pretreatment levels of neutrophils, monocytes, lymphocytes, and eosinophils in the peripheral blood were not associated with disease control; moreover, the N/L ratio did not show any association with disease control (Table 2). Posttreatment changes in these parameters, at both 28 and 42 days, were not associated with disease control (Table 2).

The baseline levels of total αβ T cells, CD4 T cells, and CD8 T cells were similar in patients with disease control and in those with disease progression (Fig. 2A–C). Patients with disease control tended to show CD8 T cells with lower PD-1 positivity than did patients with disease progression ($p = 0.065$; Fig. 2E). Additionally, patients with disease control exhibited peripheral B cells ($CD19^+$) with significantly lower PD-1 positivity than did patients with disease progression ($p = 0.042$; Fig. 2F; Supplementary Fig. 5B). The same trends were observed when only patients with treatment responses were compared with those with disease progression (Supplementary Fig. 3A, http://links.lww.com/JCMA/A68).

Posttreatment fold changes in total αβ T cells, CD4 T cells, and CD8 T cells were not associated with disease control (Fig. 3A–C). Posttreatment changes in the PD-L1 positivity of monocytes were significantly different between patients with disease control and those with disease progression, both at 28 ($p = 0.020$) and 42 ($p = 0.008$) days. Patients with disease progression were more likely to exhibit monocytes ($CD14^+$) with increased PD-L1 positivity after treatment (Fig. 3D; Supplementary Fig. 5C, http://links.lww.com/JCMA/A70). The same trend was observed when only patients with treatment responses were compared with those with disease progression (Supplementary Fig. 3B, http://links.lww.com/JCMA/A68). The pretreatment and posttreatment percentage of NK cells and CTLA-4 cells are demonstrated on Supplementary Fig. 4 (http://links.lww.com/JCMA/A69).

Table 2

Pretreatment hemogram results and their association with disease control

	Best response		
	Disease control	Disease progression	*p*
Pretreatment			
Neutrophil (/μL)	3207.8 (2646.9–4527.1)	2137.8 (1108.2–6312.7)	0.446
Monocyte (/μL)	387.9 (265.9–543.8)	255.6 (178.1–550.4)	0.521
Lymphocyte (/μL)	1133.8 (863.7–1731.1)	1332.5 (1118.9–2219.9)	0.379
Eosinophil (/μL)	131.9 (46.2–322.7)	83.3 (47.6–197.0)	0.446
N/L ratio	2.70 (2.09–4.16)	1.78 (0.63–4.31)	0.379
Day 28 compared with pretreatment			
Neutrophil (/μL)	+74.3 (–1378.6–825.5)	+293.9 (–2301.2–1027.8)	1.000
Monocyte (/μL)	–0.3 (–109.9–68.6)	+80.2 (–204.5–198.5)	0.521
Lymphocyte (/μL)	+119.1 (–150.8–221.0)	+268.6 (–104.3–657.3)	0.599
Eosinophil (/μL)	–2.3 (–48.6–34.7)	+33.7 (3.8–182.9)	0.170
N/L ratio	+0.06 (–1.46–0.24)	–0.23 (–2.27–0.96)	0.953
Day 42 compared with pretreatment			
Neutrophil (/μL)	–90.7 (–713.6–286.5)	+ 406.1 (–523.4–1452.7)	0.316
Monocyte (/μL)	–23.5 (–145.5–68.2)	+90.2 (–141.9–153.4)	0.379
Lymphocyte (/μL)	–30.5 (–234.1–188.7)	+13.7 (–220.2–358.9)	0.770
Eosinophil (/μL)	+18.5 (–16.2–44.5)	+26.1 (–24.8–181.9)	0.521
N/L ratio	–0.10 (–1.03–0.35)	+0.06 (–1.12–1.67)	0.684

Data presented as median (interquartile change).
N/L = neutrophil/lymphocyte.

4. DISCUSSION

In this study, we demonstrated that the low pretreatment PD-1 positivity of peripheral B cells and the constant posttreatment PD-L1 positivity of monocytes were associated with disease control after nivolumab treatment for advanced HCC. Although the number of patients was limited, this study is the first to identify potential peripheral blood biomarkers of anti-PD-1 or anti-PD-L1 treatment efficacy.

A recent study, which enrolled 149 patients with 15 cancer types, revealed that patients with microsatellite instability-high (MSI-H) or mismatch repair-deficient (dMMR) cancers had a higher response rate for pembrolizumab, an anti-PD-1 anitbody, therapy.[14] Subsequently, FDA first approved the tissue/site-agnostic indication for pembrolizumab in patients with MSI-H or dMMR cancers. In addition to the MSI-H or dMMR status of cancer, tumor PD-L1 expression, and mutational burden have been proposed as predictive biomarkers.[15–18] However, these biomarkers are unreliable because of intra- and intertumoral heterogeneity. Their predictive power may only be realized when conducting serial biopsy.[19] A noninvasive and precise predictive marker of immune checkpoint inhibitors is an unmet need.

In our study, the low PD-1 positivity of peripheral B cells was significantly associated with disease control. PD-1 has been reported to inhibit B cell receptor signaling and regulate B-cell activation.[20,21] A recent study showed that PD-1-expressing B cells in HCC tissues exerted a protumorigenic effect through IL-10 signaling and the suppression of tumor-specific T-cell immunity.[22] High PD-1 expression in peripheral B cells may reflect the effect of anti-PD-1 therapy, which may be related to B cells at tumor sites and inhibits T cell immunity, and as a result, eventually leads to a poor treatment response.

Increased PD-L1 expression in monocytes after treatment was associated with disease progression. PD-L1 expression in monocytes has been shown to inhibit the induction of mixed lymphocyte reactions[23] and has been proposed as a resistance mechanism for anti-CD40 therapy.[24] In the peritumor stroma of HCC, monocytes activated by tumors have been reported to strongly express PD-L1 proteins and effectively suppress tumor-specific T cell immunity.[25] The changes in peripheral monocytes may reflect the condition at tumor sites; therefore, increased PD-L1 expression in peripheral monocytes may imply that tumor-specific T-cell immunity is suppressed even after anti-PD-1 therapy.

Lymphocyte counts at baseline and their posttreatment changes are associated with the efficacy of anti-CTLA4 therapy.[26–28] Posttreatment changes in the N/L ratio and eosinophil counts have also been reported to predict treatment outcomes.[28,29] However, we did not find such associations in our study. This suggests that biomarkers of anti-PD-1 therapy are different from those of anti-CTLA4 therapy, although both are immune checkpoint inhibitors.

Our study has several limitations. First, because it was based on an ongoing clinical trial, the study had a limited sample size. Immune-checkpoint inhibitor was not yet approved as a standard therapy in hepatocellular carcinoma when the study was done. Under the ethical concern, we could only prove our concept by ongoing clinical trial. Although the population is

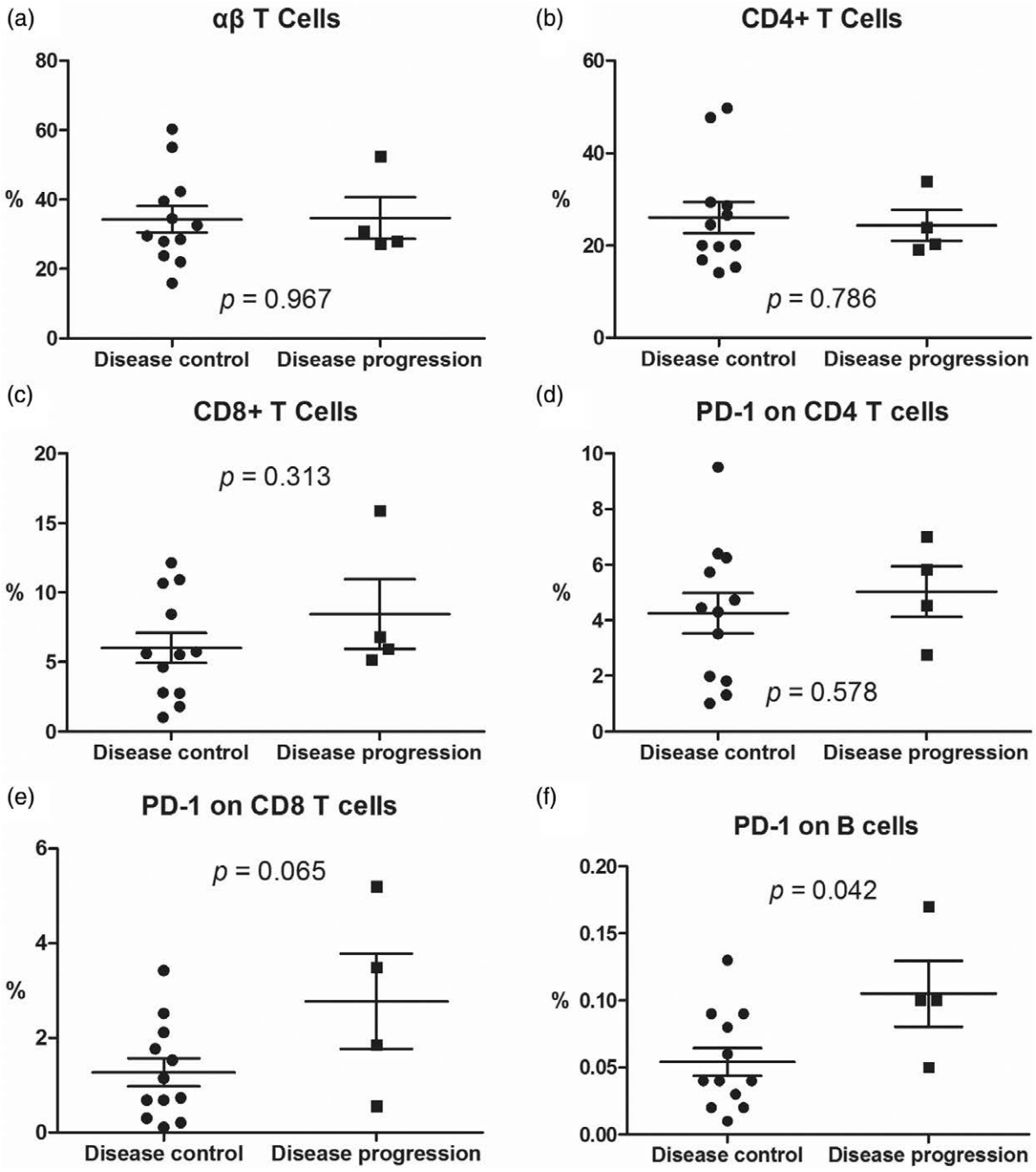

Fig. 2 Comparisons of (A) total αβ T cells, (B) CD4 T cells, (C) CD8 T cells, (D) PD-1 positivity among CD4 T cells, (E) PD-1 positivity among CD8 T cells, and (F) PD-1 positivity among B cells in patients with disease control and those with disease progression. Bars represent the standard error of the mean.

limited, we believe the data are with great quality. Second, the fluctuation in the blood cell count may be confounded by many factors, which makes analysis challenging. To correct the inter-individual variation, we carefully extract the buffy coat and we calculate the fold change for further analysis. Third, according to the clinical protocol, the blood sampling time was fixed. Thus, the earliest blood sampling time was 4 weeks. An earlier follow-up schedule may not only be more practical clinically but also ensure more sensitivity for detecting the response of the immune system to nivolumab treatment. In practice, it takes around 8 to 10 weeks to detect a clinical response in HCC patients under immunotherapy by image study such as CT or MRI.[13] In other words, it is still valuable to have a preemptive predictive marker as early as 4 weeks for clinical decision making.[30] Fifth, the chronic hepatitis B or hepatitis C may also be an impact to immune cell profiles in HCC patients. Based on the global clinical trial, CHECKMATE-040,[13] The enrollment not only exclude patients with active hepatitis and abnormal liver function but also exclude patients with HBV viral load more than 100 IU/mL. Furthermore, all patients need to be in Child-Pugh score A. Antiviral therapy needs to be prescribed in patients with hepatitis B during the whole treatment course. We believe the strict protocol can minimize the confounding effects of viral hepatitis.

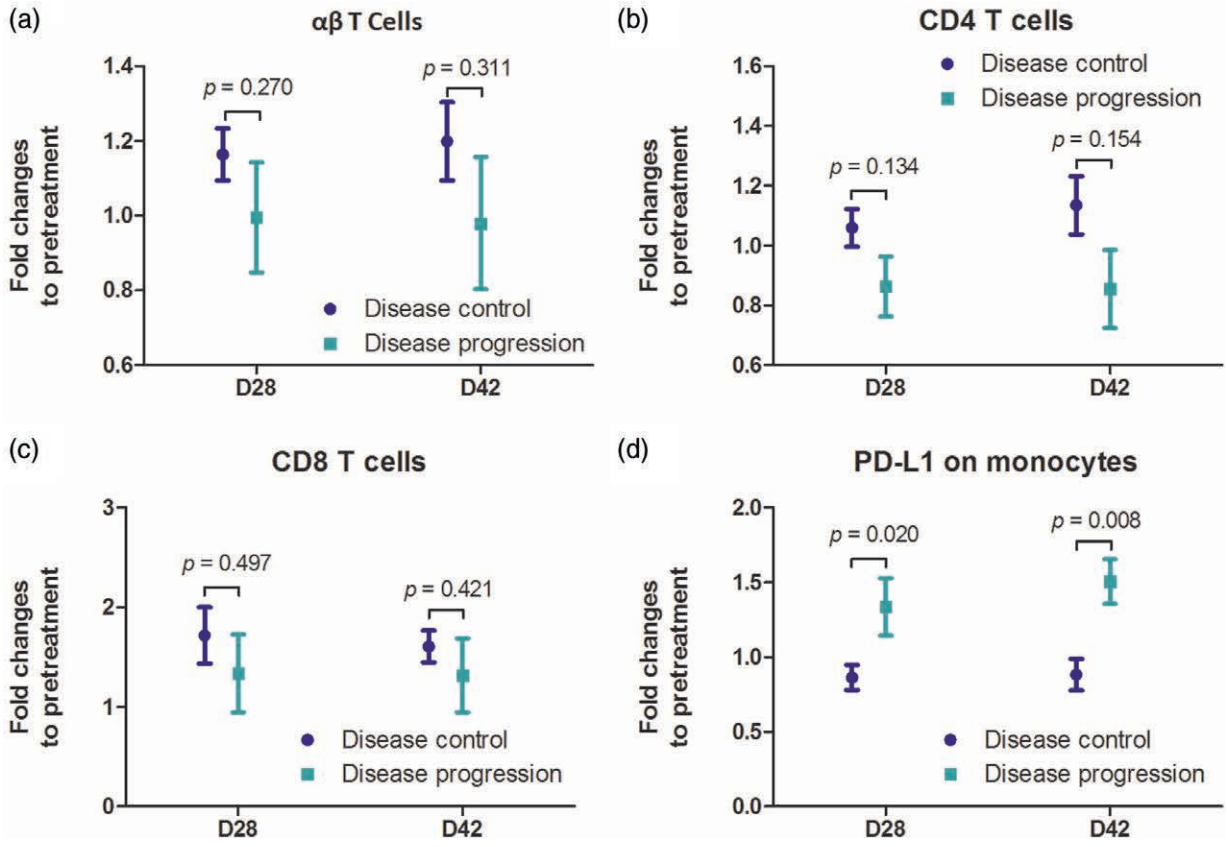

Fig. 3 Comparisons of fold changes in (A) total αβ T cells, (B) CD4 T cells, (C) CD8 T cells, and (D) PD-L1 positivity on monocytes after nivolumab treatment in patients with disease control and those with disease progression. Mean and standard error are plotted.

This is the first study to identify potential biomarkers of anti-PD-1 treatment efficacy for advanced HCC. This study investigated the dynamic changes in immune cells in patients receiving anti-PD-1 therapy. These results were correlated with the treatment outcome. Our results are hypothesis generating at best and require further validation. However, the finding regarding the post-treatment PD-L1 positivity of monocytes was confirmed at two separate time points, which reinforces the validity of the results.

In conclusion, the low pretreatment PD-1 positivity of peripheral B cells and the constant posttreatment PD-L1 positivity of monocytes were associated with disease control after nivolumab treatment for advanced HCC. These findings require confirmation in additional large prospective studies.

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APPENDIX A. SUPPLEMENTARY DATA

Supplementary data related to this article can be found at http://doi.org/10.1097/JCMA.0000000000000477.

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