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Original Article

Serum concentrations of HGF are correlated with response to anti-PD-1 antibody therapy in patients with metastatic melanoma

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ABSTRACT

Background: Anti-programmed cell death protein (PD)-1 antibody treatment is associated with a notable improvement in only 30%–40% of patients. Thus, a predictive and easily measured marker of the clinical benefit of anti-PD-1 antibody treatment is necessary; therefore, in this study, we focused on the serum concentration of hepatocyte growth factor (HGF).
Objectives: To evaluate whether the serum concentration of HGF can be used as a biomarker for the clinical response to anti-PD-1 antibody therapy.
Methods: This study included 29 metastatic melanoma patients receiving nivolumab or pembrolizumab. Nine patients responded to anti-PD-1 antibody treatment, whereas the other 20 patients did not. The serum concentrations of HGF were analyzed by using ELISA. In 28 patients, immunohistochemical analysis of the HGF protein in patients' cancer tissues was also performed. Peripheral blood mononuclear cells (PBMCs) from healthy donors were cultured with an anti-CD3 antibody in the presence or absence of HGF and c-MET inhibitor. The expression of perforin in $CD8^+$ T cells were evaluated by using flow cytometry.
Results: Among the 29 recruited patients, the non-responders displayed higher serum concentrations of HGF than the responders ($P = 0.00124$). Patients with low serum concentrations of HGF showed longer overall survival ($N = 28$, $P = 0.039$; HR 0.3125, 95% CI 0.1036–0.9427) and progression-free survival ($N = 24$, $P = 0.0068$; HR 0.2087, 95% CI 0.06525–0.6676) than those with high concentrations of HGF. We observed a significant correlation between the serum concentration of HGF and immunohistochemical-positive staining ($P = 0.000663$). In a flow cytometry analysis of PBMCs from healthy donors, HGF was found to downregulate perforin secretion. Furthermore, the addition of capmatinib, a specific inhibitor of c-MET, increased the expression of perforin in $CD8^+$ T cells.
Conclusions: HGF concentration represents a valid biomarker that can be further developed for the evaluation of anti-PD-1 therapy. Our results suggested that c-MET inhibition promotes perforin expression in $CD8^+$ T cells. Therefore, c-MET inhibitors can activate the immune system and may play an important role in combined immunotherapy.

1. Introduction

Immune checkpoint blockades have improved the overall survival of metastatic melanoma patients [1–3]. Anti-programmed cell death protein (PD)-1 antibodies, such as nivolumab or pembrolizumab, block PD-1, an inhibitory immune checkpoint receptor expressed on activated T cells. Nivolumab is associated with a significant improvement in overall survival and progression-free survival, and 30%–40% of patients experience long-term benefits; similar findings are observed for pembrolizumab [4,5]. Although anti-PD-1 antibodies are highly efficient, this treatment is very costly. The expression of PD ligand 1 (PD-L1) in tumor cells is associated with reactive properties to the blockade of this immune checkpoint [6,7]; however, the objective measurement of

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PD-L1 protein concentration reveals heterogeneity within tumors and large inter-assay variability or discrepancies. In the treatment of non-small-cell lung cancer, PD-L1 expression in at least 50% of tumor cells was correlated with the improved efficacy of pembrolizumab [8]. However, no conclusive data exist to support similar conclusions in melanoma. Ribas et al. indicated that tumor regression after therapeutic PD-1 blockade required pre-existing $CD8^+$ T cells [9]. However, in the method used, a certain volume of metastatic tumors is needed. Methods for the measurement of PD-L1 expression are also inaccurate because of the heterogeneity of PD-L1 expression. Therefore, a simpler prediction method, such as a serum marker for the clinical benefit of anti-PD-1 antibody is needed. Cytokines, broadly defined as secreted cell-cell signaling proteins distinct from classic hormones or neurotransmitters, play important roles in inflammation, innate immunity, apoptosis, angiogenesis, cell growth, and differentiation, and are reported to be involved with numerous diseases, including cancer, obesity, and inflammatory and cardiac diseases.

Here, we focused on the serum concentration of hepatocyte growth factor (HGF) from cytokine antibody array data. HGF was purified from the plasma of a patient with fulminant hepatic failure [10]; Miyazawa et al. [11] and Nakamura et al. [12] sequenced cDNAs encoding HGF. Kilby et al. found that the protein and mRNA expression of both hepatocyte growth factor and its receptor (c-MET) were present in third trimester placentas, which suggested that HGF serves as a paracrine mediator to control placental development and growth [13]. In addition, HGF also controls the growth, invasion, and metastasis of cancer cells, with activating c-Met mutations predisposing humans to cancer [14]. The serum concentration of HGF was reported as a poor prognostic factor in different types of cancer, such as lung cancer, gastric cancer, colon cancer, and melanoma [15]. It has been reported that HGF is involved in malignant alteration through the proliferation of tumor cells and neoangiogenesis [16] via the HGF/MET pathway [17,18], whereas the HGF-producing cells and the biological activity of HGF remain unknown.

In this study, we evaluated whether the serum concentration of HGF can be used as a biomarker of anti-PD-1 antibodies. We also focused on the effect of HGF on $CD8^+$ T cells. We examined how the activity of $CD8^+$ T cells was altered after the inhibition of the HGF/MET pathway.

2. Materials and methods

2.1. Patients, treatment, and clinical evaluation

This study included 29 patients (16 men and 13 women) with metastatic melanoma patients receiving nivolumab or pembrolizumab at Kumamoto University Hospital, with age range of 28–83 years (mean = 64 years). This study was approved by the ethical committee of the Kumamoto University Graduate School of Medicine. The patients were included if they had a confirmed diagnosis of stage III or IV unresectable melanoma, according to the 2009 American Joint Committee on Cancer melanoma staging and classification. The other inclusion criteria were: at least 20 years of age; no specific melanoma therapy within the previous 28 days. Patients with all histological types of melanoma, including mucosal and uveal melanoma, were eligible for inclusion. The exclusion criteria were: presence of an autoimmune disease, HIV, hepatitis B or C, pregnancy, hepatic metastasis, renal failure or concomitant chemotherapy, or any history of prior immunotherapy for melanoma.

For comparison, 44 age-matched healthy controls and 108 patients with melanoma recruited. Of the 108 melanoma patients, 15, 14, 25, 22, and 32 were diagnosed with stage 0, I, II, III, and IV melanoma, respectively. Primary melanomas were classified into four clinical and pathological subtypes: (i) melanoma arising from non-cumulative sun-induced damaged skin (non-CSD); (ii) cumulative sun-induced damaged skin (CSD); (iii) acral melanoma; and (iv) mucosal melanoma: 28 patients had non-CSD, 13 patients had CSD, 43 patients had acral melanoma, and 19 had mucosal melanoma. Treatment efficacy was assessed by contrast-enhanced computed tomography (CT), magnetic resonance imaging, or positron emission tomography-CT (PET-CT) after the third infusion of anti-PD-1 antibody and the clinical response was defined based on Response Evaluation Criteria in Solid Tumors (RECIST; Version 1.1). The clinical response was defined as complete response (CR), partial response (PR), stable disease (SD), or progressive disease (PD). We defined patients with CR and PR as responders and patients with SD and PD as non-responders. Therefore, among the 29 recruited patients, 9 patients responded to anti-PD-1 antibody treatment, whereas the other 20 patients did not.

2.2. Cytokine antibody arrays

Pretherapeutic serum concentrations of 120 cytokines were analyzed by using cytokine antibody arrays (RayBio Human Cytokine Antibody Array G-Series 1000, RayBiotech, Norcross, GA, USA) in six patients with melanoma (three responders and three non-responders) before the first treatment with an anti-PD-1 antibody.

2.3. ELISA

A Human HGF ELISA Kit (RayBiotech, Norcross, GA, USA) was used in accordance with the manufacturer's instructions. The absorbance at 450 nm was measured by using a microplate reader (EZS-ABS, IWAKI, Tokyo, Japan).

2.4. Immunohistochemical analysis

Immunohistochemical analysis of the HGF protein in the cancer tissues of patients was performed as described previously [19] using a rabbit monoclonal antibody against HGF [EPR12230] (1:100 dilution; Abcam, Cambridge, UK). An isotype monoclonal rabbit antibody (Nichirei, Tokyo, Japan) was used as a negative control.

Heavily pigmented melanoma lesions are difficult to evaluate with routine hematoxylin and eosin staining because it is difficult to distinguish pigmented melanocytes from the numerous melanophages that are usually seen in the background of these lesions. Immunoperoxidase staining for the HGF antibody using diaminobenzidine (DAB) as the chromogen, which forms a brown product, does not adequately distinguish melanocytes from melanophages. We modified this technique by replacing hematoxylin as the counterstain with azure B (Giemsa stain), which stains melanin green-blue. Thus, positively stained melanocytes appear brown, whereas the melanin granules in their cytoplasm are green-blue [20].

The slides were mounted in aqueous medium and viewed under a microscope. The staining intensity was classified as follows: strong plasmalemmal immunostaining (staining intensity score = 3); weak plasmalemmal immunostaining (staining intensity score = 2); nonspecific immunostaining (staining intensity score = 1); or negative immunostaining (staining intensity score = 0). Nonspecific immunostaining was the same intensity as the staining intensity in the adjacent epidermis. We categorized the samples into a positive group (scores of 2 and 3) and a negative group (scores of 0 and 1), and evaluated the correlation between the serum concentration of HGF and the intensity of staining. The samples were evaluated independently by two blinded observers (Y.K. and S.F.).

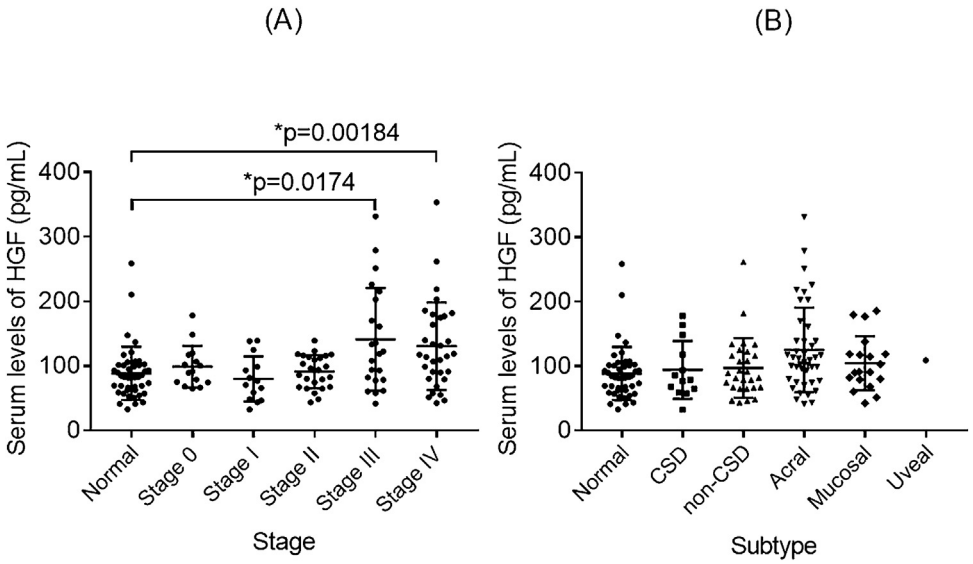

Fig. 1. Serum concentrations of HGF in melanoma patients in each stage and subtype (A)(B).
(A) Stage: Stage I–IV patients and normal control group.
(B) Subtype: CSD, non-CSD, acral, mucosal, uveal, and normal control group.
CSD; cumulative sun-induced damaged skin non-CSD; non-cumulative sun-induced damaged skin.

2.5. Human HGF stimulation and blocking assay

PBMCs from healthy donors were cultured with anti-CD3 antibody (0.5 mg/mL, BioLegend, San Diego, CA, USA) for 72 h in the presence or absence of HGF (20 ng/mL) and capmatinib (also known as INCB28060 and INC280) (Selleckchem.com, Houston, TX, USA) (50 ng/mL). For the intracellular staining of cytokines, PBMCs were stimulated for 4 h with BD Leukocyte Activation Cocktail (ionomycin, brefeldin A, and phorbol myristic acetate (PMA)). To detect intracellular protein, the cells were permeabilized, fixed, and stained in accordance with the manufacturer's instructions using the IntraPrep Permeabilization Reagent kit (Beckman Coulter, Beckman Coulter, Marseille, France). The intracellular expression levels of perforin and c-MET of $CD8^+$ T cells were evaluated by flow cytometry (EasyCyte Mini flow cytometer, Merck Millipore, Billerica, MA, USA, and NovoCyte Flow Cytometer, ACEA Biosciences Inc., San Diego, CA, USA).

2.6. Statistical analysis

Unless otherwise indicated, the data are presented as the mean ± standard deviation (SD) and are representative of three independent experiments. The *P*-values were calculated by using the Mann-Whitney U test or the log-rank test; values of less than 0.05 were considered statistically significant and are denoted by

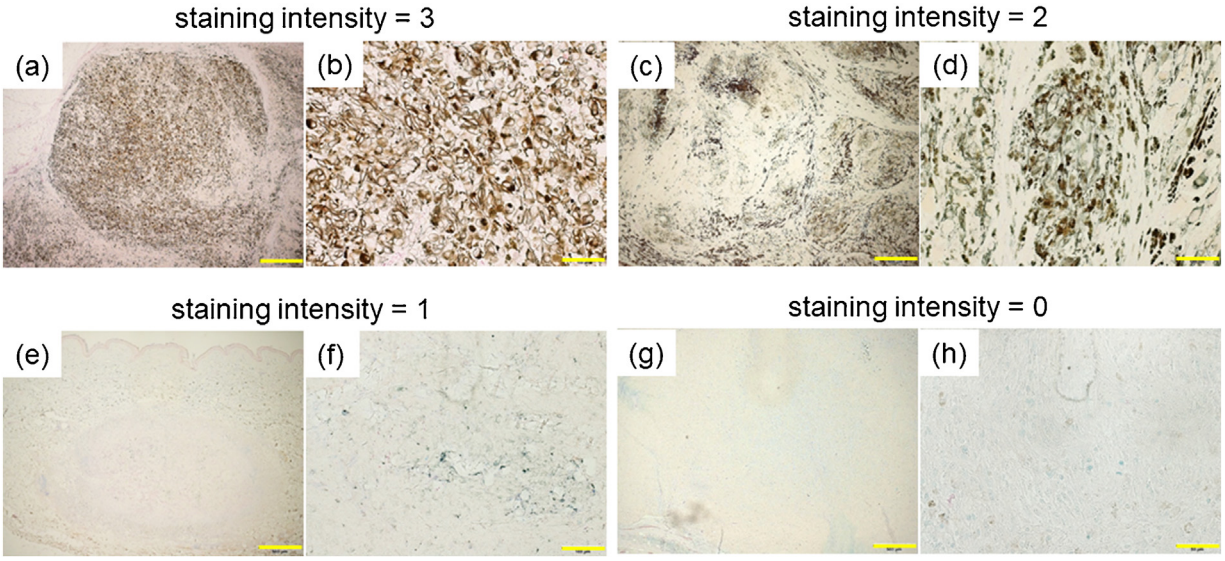

Fig. 2. Representative images of typical immunohistochemical staining for HGF in metastatic malignant melanoma (a–h).
a, b; strong immunostaining (staining intensity score =3),
c, d; moderate immunostaining (staining intensity score =2),
e, f; weak immunostaining (staining intensity score =1),
g, h; negative immunostaining (staining intensity score =0),
Bar = 500 μm (a, c, e, and g), 100 μm (b, d, g, and h).

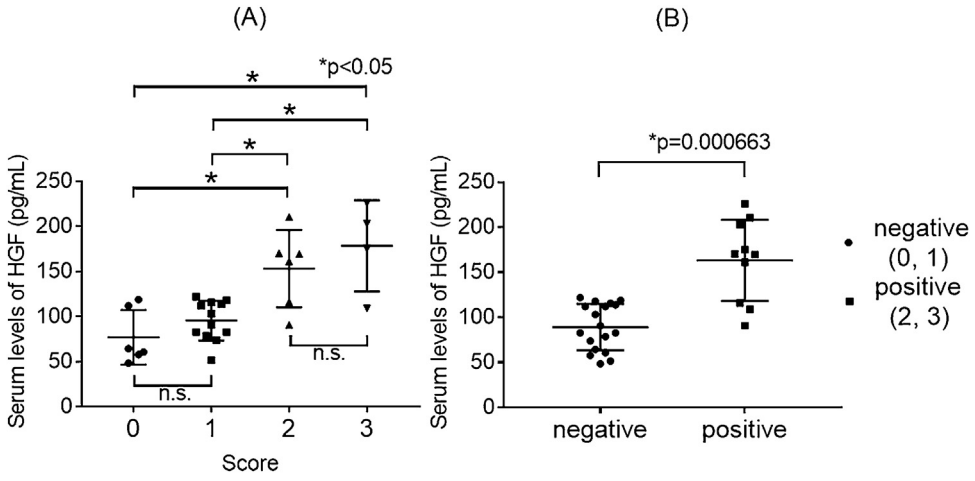

Fig. 3. The correlation between the serum concentration of HGF and immunohistochemical staining.
The scores of 0 and 2, 0 and 3, 1 and 2, 1 and 3 groups were significantly different respectively (Fig. 3A). The serum concentration of HGF in the positive group (scores 2 and 3) were significantly higher than those in the negative group (scores 0 and 1) (P = 0.000663).

asterisks (*) in the figures. The Kaplan-Meier method was used to obtain estimates of OS and PFS; we compared curves with a two-tailed log-rank test. PFS was measured from the date treatment started to the date of documented progression or death. The patients who were alive and not known to have progressed were censored. OS was measured from the date treatment started to the date of death or last follow-up. The multivariate analyses were performed using the Cox proportional hazards model to identify independent predictors of OS and PSF. Statistical analyses were computed by using SAS Statistical Software Version 9.4 (SAS Institute Inc., Cary, NC). A P-value of less than 0.05 was considered statistically significant.

3. Results

To identify candidate biomarkers for anti-PD-1 therapy, pretherapeutic serum concentrations of 120 cytokines were assessed in six patients with melanoma (three responders and three non-responders) before the first treatment with anti-PD-1 antibody (Supplementary Tables 1 and 2). Among the candidates, we focused on serum HGF concentration, which was significantly low in three responders (P = 0.00919). We measured the serum concentration of HGF in patients with melanoma by ELISA and investigated the relationship between the stages (Stage 0–IV) and melanoma subtypes (CSD, non-CSD, acral, mucosal, and uveal) compared with normal controls. The serum concentrations of HGF in patients with stage III and IV melanoma were significantly higher than those in the normal controls. In contrast, there was no difference in serum HGF concentration in each subtype of melanoma (Fig. 1). Peripheral blood was collected for up to 7 days before the first anti-PD-1 antibody infusion ("pre") and, if possible, approximately 3 months after the first treatment ("post").

Next, we compared the serum concentration of HGF and HGF expression in tumor specimens through the scoring intensity of immunohistochemical staining. Some immunocompetent cells, such as monocytes, were also stained [21,22]. Representative images of typical immunohistochemical staining for HGF in metastatic malignant melanoma are shown in Fig. 2. We divided the samples into four groups based on the staining properties and scores: strong plasmalemmal immunostaining (staining intensity score = 3); weak plasmalemmal immunostaining (staining intensity score = 2); nonspecific immunostaining (staining intensity score = 1); and negative immunostaining (staining intensity score = 0). The scores of 0 and 2, 0 and 3, 1 and 2, 1 and 3 groups were significantly different, respectively (Fig. 3A). In addition, the serum concentration of HGF in the positive group (scores 2 and 3) were significantly higher than those in the negative group (scores 0 and 1) (P = 0.000663) (Fig. 3B).

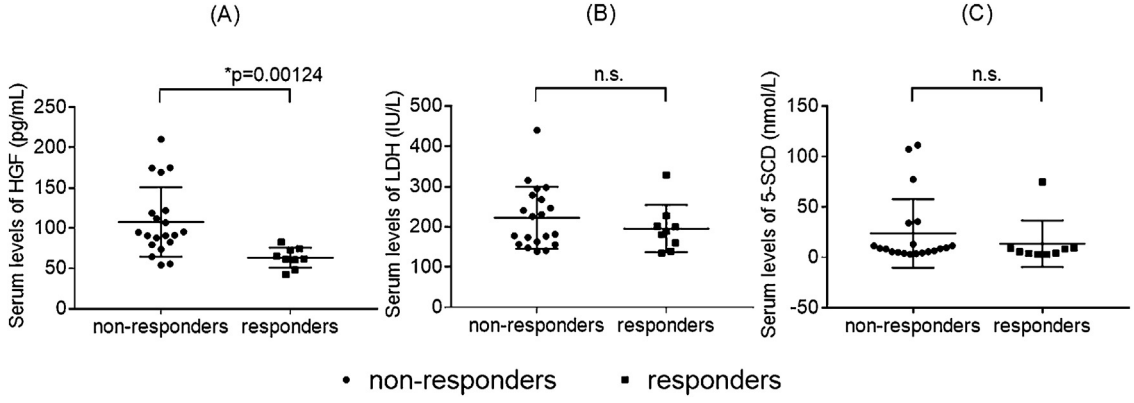

Fig. 4. The serum concentration of hepatocyte growth factor (HGF) (A), lactate dehydrogenase (LDH) (B), and 5-S-cysteinyldopa (5-S-CD) (C) in non-responders and responders to anti-PD 1 therapy.

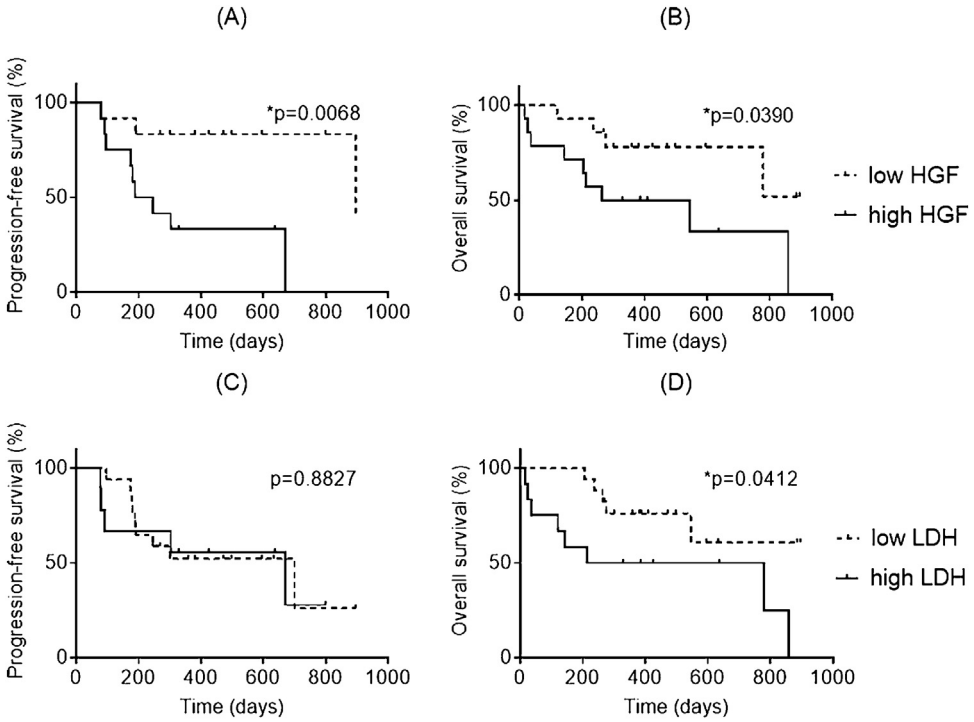

Fig. 5. The relationship between the serum concentration of HGF and overall survival or progression-free survival.
(A) Kaplan-Meier curve show the PFS of patients with above (high) or below (low) the median of baseline serum concentration of HGF (N = 24, P = 0.0068; HR 0.2087; 95% CI 0.06525–0.6676). (B) Kaplan-Meier curves showing the OS of patients with above (high) or below (low) the median of baseline serum concentration of HGF (N = 28, P = 0.039; HR 0.3125; 95% CI 0.1036–0.9427).
The relationship between the serum concentration of LDH and overall survival or progression-free survival.
(C) Kaplan-Meier curve show the PFS of patients with above (high) or below (low) upper limit of normal serum level of LDH (N = 26, P = 0.8827; HR 0.9217, 95% CI 0.3047–2.788). (D) Kaplan-Meier curves showing OS of patients with above (high) or below (low) the upper limit of normal serum level of LDH (N = 29, P = 0.0412; HR 0.3333, 95% CI 0.1065–1.044).

Next, we compared serum HGF concentrations in responders and non-responders. The non-responders had elevated serum concentrations of HGF compared with the responders (P = 0.00124). The serum concentrations of 5-*S*-cysteinyldopa, a tumor marker for melanoma, and lactate dehydrogenase did follow this tendency. In addition, we divided the patients into two groups based on median serum HGF concentration and assessed PFS and OS (Fig. 4). The Kaplan-Meier curves showed that the patients with low serum concentrations of HGF showed longer PFS (N = 24, P = 0.0068; HR 0.2087; 95% CI 0.06525–0.6676) and OS (N = 28, P = 0.039; HR 0.3125; 95% CI 0.1036–0.9427) than those with high concentrations of HGF, respectively (Fig. 5A, B). Next, we divided the patients into two groups by upper limit of normal serum level of LDH and assessed PFS and OS. Kaplan–Meier curves showed that patients with low serum levels of LDH showed longer OS than those with high levels (N = 29, P = 0.0412; HR 0.3333, 95% CI

Table 1
Univariate Cox proportional hazard models of progression-free and overall survival.

	PFS				OS			
	HR	95%CI		p value	HR	95%CI		p value
LDH low	1.00			0.8827	1.00			0.0519
LDH high	1.09	0.36	3.28		3.06	0.991	9.416	
LDH (per unit)	1.00	0.99	1.01	0.9579	1.01	1.004	1.02	0.0023
HGF low	1.00			0.013	1.00			0.0378
HGF high	4.50	1.37	14.76		3.52	1.073	11.522	
HGF (per unit)	1.02	1.01	1.04	0.0021	1.02	1.006	1.033	0.0049
PS 0	1.07	0.23	4.91	0.928	*			**
1	*				0.00	0	0	
4	1.00				1.00			
Stage 3b	0.00	0.00		0.8845	0.00	0		0.8267
3c	0.68	0.15	3.09		0.62	0.134	2.847	
4	1.00				1.00			

HR : hazard ratio, 95%CI : 95% confidence interval.
*: HR estimate was not available.
**: not assessable.

0.1065–1.044). But Kaplan–Meier curves did not show significant difference between the two groups in PFS (N = 26, P = 0.8827; HR 0.9217, 95% CI 0.3047–2.788) (Fig. 5C, D).

We conducted multivariate analysis, including other possible factors associated with survival, such as serum LDH above the normal range (and per unit), performance status (0–4), and metastatic stage (unresectable III/ IV), because HGF may just reflect the general condition of the patients. In the univariate Cox proportional hazard models, high concentrations of HGF and actual HGF value (per unit) are significant adverse prognostic factors of PFS, whereas the actual LDH value (per unit), the high concentrations of HGF and actual HGF value (per unit) were significant adverse prognostic factors of OS (Table 1). In the next phase, multivariate analyses were performed by using the Cox proportional hazards model to identify independent predictors of PFS and OS. The serum concentration of HGF per unit was the most significant independent predictor of PFS (P = 0.0021), and serum

Table 2
Multivariate Cox proportional hazard models of progression-free and overall survival.

	PFS				OS			
	HR	95%CI		p value	HR	95%CI		p value
LDH (per unit)	1.00	0.99	1.01	0.9131	1.02	1.01	1.03	0.0016
HGF (per unit)	1.02	1.01	1.04	0.0021	1.02	1.01	1.04	0.0028

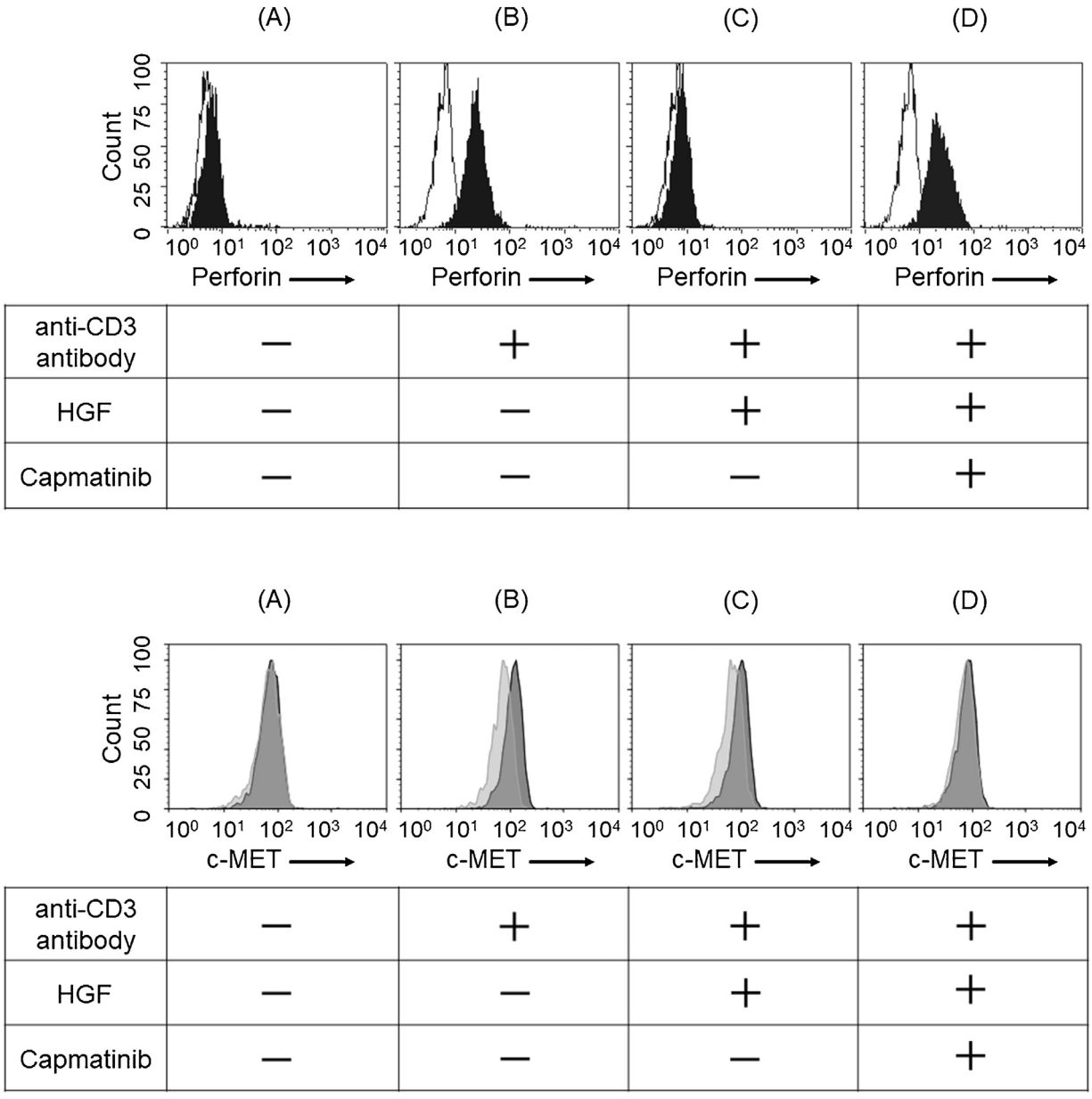

Fig. 6. The histogram shows the expression of intracellular perforin (Fig. 6-1) and cell-surface c-MET (Fig. 6-1) in CD8$^+$ T cells in the presence or absence of the c-met inhibitor, capmatinib.
The histogram shows the expression of intracellular perforin (Fig. 6-1) and cell-surface c-MET (Fig. 6-2) in CD8$^+$ T cells in the presence or absence of the c-met inhibitor, capmatinib.
(A) PBMCs alone cultured for 72 h.
(B) PBMCs cultured for 72 h with anti-CD3 antibody.
(C) PBMCs cultured with anti-CD3 antibody and HGF.
(D) PBMCs cultured with anti-CD3 antibody, HGF, and capmatinib.
In each figure, the histogram on the extreme left is the isotype control.

concentrations of LDH per unit was the most significant independent predictor of OS (P=0.0016) (Table 2).

We also investigated chronological changes in the serum concentration of HGF, but found no correlation with the values before and after anti-PD-1 antibody therapy in a comparison between responders and non-responders (Supplementary Fig. 1).As described above, the serum concentration of HGF was suggestive of a possible beneficial effect of anti PD-1 antibody therapy.

To explain the function of serum HGF, we conducted *in vitro* experiments. We evaluated the effect of HGF on perforin expression in $CD8^+$ T cells *in vitro*. Human PBMCs were cultured in the presence or absence of HGF, and the expression of perforin (Fig. 6-1) and c-MET (Fig. 6-2) were evaluated by using flow cytometry. Anti-CD3 antibody increased the expression of perforin in $CD8^+$ T cells (Fig. 6-1B). The expression of perforin in $CD8^+$ T cells was reduced in the presence of HGF (Fig. 6-1C). The receptor of HGF was previously reported as c-Met [23]. The c-Met inhibitor capmatinib prevented the suppressive effects of HGF (Fig. 6-1D). In the presence of the anti-CD3 antibody the cell-surface expressions of c-MET in $CD8^+$ T cells were significantly increased. The MFIs (mean ± SD) for the mAb c-MET and the isotype control were 102.7 ± 2.2 and 73.3 ± 2.5, respectively ($P < 0.01$) (Fig. 6-2B). The expressions of c-MET in $CD8^+$ T cells were not changed by the addition of HGF (Fig. 6-2C). However, in the presence of capmatinib, the cell-surface expressions of c-MET in $CD8^+$ T cells were undetectable (Fig. 6-2D). These data suggested that the function of cytotoxic T cells was suppressed by HGF and that the inhibitor, capmatinib, recovered this immunosuppression.

4. Discussion

HGF was first purified from the plasma of a patient with fulminant hepatic failure, which has been demonstrated to have a major role in embryonic organ development. HGF regulates cell growth, cell motility, and morphogenesis through the activation of a tyrosine kinase signaling cascade after binding to the proto-oncogenic c-Met receptor. HGF is secreted by mesenchymal cells and acts as a multifunctional cytokine on cells mainly epithelial in origin. Its ability to stimulate mitogenesis, cell motility, and matrix invasion ensures it has central roles in angiogenesis, tumorigenesis, and tissue regeneration. HGF controls growth, invasion, and metastasis in cancer cells, and activating c-Met mutations predispose humans to cancer. In fact, the serum concentration of HGF was reported as a poor prognostic factor in different types of cancer (e.g., lung cancer, gastric cancer, colon cancer, and melanoma). HGF is involved in malignant alterations through proliferation of tumor cells and neoangiogenesis via the HGF/MET pathway.

Capmatinib (also known as INCB28060 and INC280) is a potent, orally bioavailable, ATP-competitive inhibitor of c-MET, with an IC_{50} of 0.13 nM in a cell-free assay; it is inactive against RONβ, epidermal growth factor receptor, and HER-3. Capmatinib selectively binds to c-Met, which inhibits c-Met phosphorylation and disrupts the c-Met signal transduction pathways. This may induce cell death in tumor cells overexpressing the c-Met protein or expressing constitutively activated c-Met protein. Collectively, capmatinib is a highly selective and potent c-MET inhibitor, and the influence of serum HGF can be easily evaluated.

In this study, we demonstrated that HGF in peripheral blood was significantly higher in non-responders to anti-PD-1 antibody treatment. Recent studies showed that genetic markers may be useful as biomarkers for immune checkpoint blockades in the treatment of melanoma [24,25]. However, no serum cytokines have yet been reported as useful and effective biomarkers. In conclusion, we have presented data that showed that the serum concentration of HGF can be a biomarker for anti-PD-1 therapy. There were some limitations to the present study; primarily, the small number of patients. To determine whether serum concentration of HGF can be used as a biomarker for anti-PD-1 therapy, further studies with larger numbers of patients are required. Second, the HGF inhibitor prevented the immunosuppression of HGF. The combination therapy of anti-PD-1 antibody and HGF inhibitor may be effective. This should also be investigated in future studies.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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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.jdermsci.2018.10.001.

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