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Clinical significance of programmed death-1 and programmed death-ligand 1 expression in the tumor microenvironment of clear cell renal cell carcinoma

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Abstract

Recently, immunotherapy based on blocking immune checkpoints with programmed death-1 (PD-1) or PD-ligand 1 (PD-L1) Abs has been introduced for the treatment of advanced clear cell renal cell carcinoma (ccRCC), especially tumors resistant to vascular endothelial growth factor-tyrosine kinase inhibitors (VEGF-TKIs), but the significance of their expression in the tumor microenvironment is unclear. We investigated these immune checkpoint markers in tumor cells and tumor-infiltrating immune cells (TIIC) in the tumor microenvironment of 100 untreated and 25 VEGF-TKI-treated primary ccRCC tissues. Upregulated expression of PD-1 and PD-L1 by TIIC, and PD-L1 by tumor cells was associated with the histological grade and unfavorable prognosis of RCC patients. High PD-1 and PD-L1 expression by TIIC was associated with a poorer response to VEGF-TKI, whereas PD-L1 expression by tumor cells did not affect the efficacy of the treatment. Furthermore, increased PD-1-positive TIIC and PD-L1-positive TIIC were observed in tumors treated with VEGF-TKIs compared

Abbreviations: ccRCC, clear cell renal cell carcinoma; indel, insertion and deletion; PD-1, programmed death-1; PD-L1, programmed death ligand-1; PR, partial response; SD, standard disease; TIIC, tumor-infiltrating immune cell; TKI, tyrosine kinase inhibitor; VEGF, vascular endothelial growth factor.

with those in untreated tumors. Our data suggest that PD-1 and PD-L1 expression by TIIC in the tumor microenvironment is involved in treatment resistance, and that sequential therapy with immune checkpoint inhibitors could be a promising therapeutic strategy for ccRCC resistant to VEGF-TKI treatment.

KEYWORDS
PD-1, PD-L1, renal cell carcinoma, tumor microenvironment, VEGF-TKI

1 | INTRODUCTION

Vascular endothelial growth factor-tyrosine kinase inhibitors, including sunitinib and sorafenib, are clinically used for advanced ccRCC, and they are thought to exert therapeutic effects on ccRCC by antagonizing the VEGF receptor, leading to reduced angiogenesis.[1] Although VEGF-TKI treatment is considered to be superior to conventional immunotherapy,[2] resistance to therapy is commonly observed in most ccRCC patients treated with VEGF-TKI within 1 year after treatment.[2] Several molecular mechanisms for resistance are suggested such as the tumor immune escape mechanism, cancer stem cells, epithelial mesenchymal transition, and reactivation of angiogenesis.[1,3-5] Among them, the tumor immune escape mechanism is considered to be one of the hallmarks of cancer because cancer cells need to escape from destruction by the immune system to survive.[6]

Programmed death-ligand 1 is known to be expressed by both TIIC in the tumor microenvironment and tumor cells, and it engages PD-1 on T cells, triggering inhibitory signaling of the T-cell receptor, thereby blocking effector functions and reducing T cell killing capacity.[7] Clinical studies investigating the effects of PD-1/PD-L1 blockade have yielded promising results in patients with advanced melanoma and other cancers, including RCC.[8] As not all patients respond to PD-1/PD-L1 blockade therapy, it is important and necessary for treatment selection to predict the likelihood of response to therapy. Therefore, information from the tumor microenvironment is important because it represents an excellent opportunity for ccRCC patients to have access to new drugs and for clinicians to find prognostic and predictive immune biomarkers. However, the focus of analyses of PD-L1 in RCC is usually on its expression by tumor cells,[9-11] and the significance of PD-L1 expression by TIIC is unclear.[10]

Certain subsets of cancer patients respond poorly to targeted therapies, probably due to the regional heterogeneity of target molecules, and immunotherapy is no exception. Expression of PD-1 and PD-L1 might have more regional heterogeneity than other mutation alterations because the PD-1/PD-L1 axis is part of a dynamic immune reaction. Indeed, recent studies suggest that the value of PD-L1 immunohistochemistry as a predictive and prognostic marker is debatable because of frequent heterogeneity.[12,13] Therefore, the regional heterogeneity of PD-1 and PD-L1 expression should be evaluated in detail in ccRCC tissues. To the best of our knowledge, however, there has been no report on the regional heterogeneity of PD-1 and PD-L1 expression on TIIC in the tumor microenvironment of ccRCC tissues. The present study was carried out to investigate the clinical relevance of PD-1 and PD-L1 expression by TIIC in the tumor microenvironment of ccRCC tissues, focusing on the heterogeneity of PD-1 and PD-L1 expression by TIIC, and its association with VEGF-TKI treatment.

2 | MATERIALS AND METHODS

2.1 | Clear cell RCC tissues without pretreatment

Total or partial nephrectomy specimens were obtained from 100 primary RCC patients without pretreatment from 1994 to 2014 at Keio University Hospital (Tokyo, Japan), and were used in the present study. This study was undertaken after approval by the Institutional Review Board of Keio University Hospital, and informed consent for the experimental use of the samples was obtained from the patients according to the hospital's ethical guidelines. Hematoxylin-eosin-stained ccRCC samples were reviewed by a well-experienced pathologist (S.M.) who is board certified and specializes in genitourinary malignancies. One representative paraffin block for each patient was selected by observing H&E-stained sections, and paraffin sections on aminopropyltrimethoxysilane-coated slides were used for immunohistochemistry. The UICC TNM system was used for tumor staging,[14] and nuclear grading was carried out according to the WHO/International Society of Urological Pathology grading system.[15]

2.2 | Clear cell RCC tissues treated with VEGF-TKIs

After approval by the Institutional Review Board of each participating institution, informed consent for the experimental use of the primary ccRCC tissues treated with VEGF-TKIs was obtained from the patients according to ethical guidelines. Pathological slides of 25 primary ccRCC tissues treated with VEGF-TKIs (7 sorafenib and 18 sunitinib) were sent for central review and evaluated by a single uropathologist (S.M.). Sorafenib or sunitinib was administered according to the protocol described previously,[2,16] and the effects were assessed according to RECIST.[17] The clinical features studied for ccRCC treated with VEGF-TKIs included age, sex, histology, and response to the therapy. No patient achieved complete response. Among 25 primary ccRCC patients pretreated with VEGF-TKI, PR was observed in 6 patients and 17 patients achieved SD. Progressive disease was observed in the other 2 patients.

2.3 | Immunohistochemistry

Paraffin sections were immunostained with anti-PD-1 goat polyclonal Ab (5 μg/mL, R&D Systems) or anti-PD-L1 rabbit mAb (Clone: E1L3N, 1:200 dilution; Cell Signaling Technology, Danvers, MA, USA) on an automated staining platform (Benchmark; Ventana, Tucson, AZ, USA)[18] with signal visualization by diaminobenzidine, and sections were counterstained with hematoxylin. For positive controls, tonsil tissue was used, as it is known to be positive for PD-1 and PD-L1. For negative controls, tissues were incubated with non-immune goat IgG or rabbit IgG (Sigma-Aldrich, St. Louis, MO, USA) at the same concentration used for each Ab.

The slides were then reviewed by an uropathologist (S.M.) blinded to the clinical information. Expression of PD-1 or PD-L1 was evaluated by counting the number of PD-1 or PD-L1-positive TIIC, which has been reported as a simple method with high reproducibility.[19] Expression of PD-1 and PD-L1 by TIIC was evaluated at the tumor nest (tumor tissue) and tumor periphery (adjacent nontumor tissue) separately.[19] The tumor periphery is the area around and adjacent to the tumor nest. All sections were reviewed at low magnification (40×) in order to detect representative areas at the tumor nest and tumor periphery separately. The average count from the 3 selected fields (high-power fields, 400×) was regarded as the PD-1- or PD-L1-positive TIIC score. For tumor cells, the proportion of PD-L1-positive cells was estimated as the percentage of total tumor cells showing membranous staining, and it was regarded as the PD-L1-positive tumor score.[10]

2.4 | Statistical analysis

The unpaired *t* test was used to analyze the relationships between the PD-1-positive TIIC score, PD-L1-positive TIIC score, or PD-L1-positive tumor score and clinicopathological parameters. Statistical analysis of the ccRCC tissues without pretreatment was carried out by dividing them into the following groups: groups of low stage (pT1 and pT2) and high stage (pT3 and pT4) or groups of low grade (grades 1 and 2) and high grade (grades 3 and 4). Receiver operating characteristic curve analysis was undertaken to determine the area under the curve, and the optimal cut-off value was taken as the farthest point from the diagonal line of the curve.[4] Cases in which the PD-1-positive TIIC score, PD-L1-positive TIIC score, or PD-L1-positive tumor score was higher than the cut-off values were defined as high cases, and those with percentages lower than the cut-off values were defined as low cases. The log-rank test and Kaplan-Meier method were used for survival analyses. Differences among groups were regarded as significant when *P* values were less than 0.05. These analyses were carried out using IBM SPSS 24, Windows version (IBM, Armonk, NY, USA).

3 | RESULTS

3.1 | Expression of PD-1 and PD-L1 in the tumor nest and tumor periphery of ccRCC without pretreatment, and its association with clinicopathological parameters

We investigated PD-1 and PD-L1 expression by TIIC at the tumor nest and tumor periphery. In low-grade ccRCC, no or very few

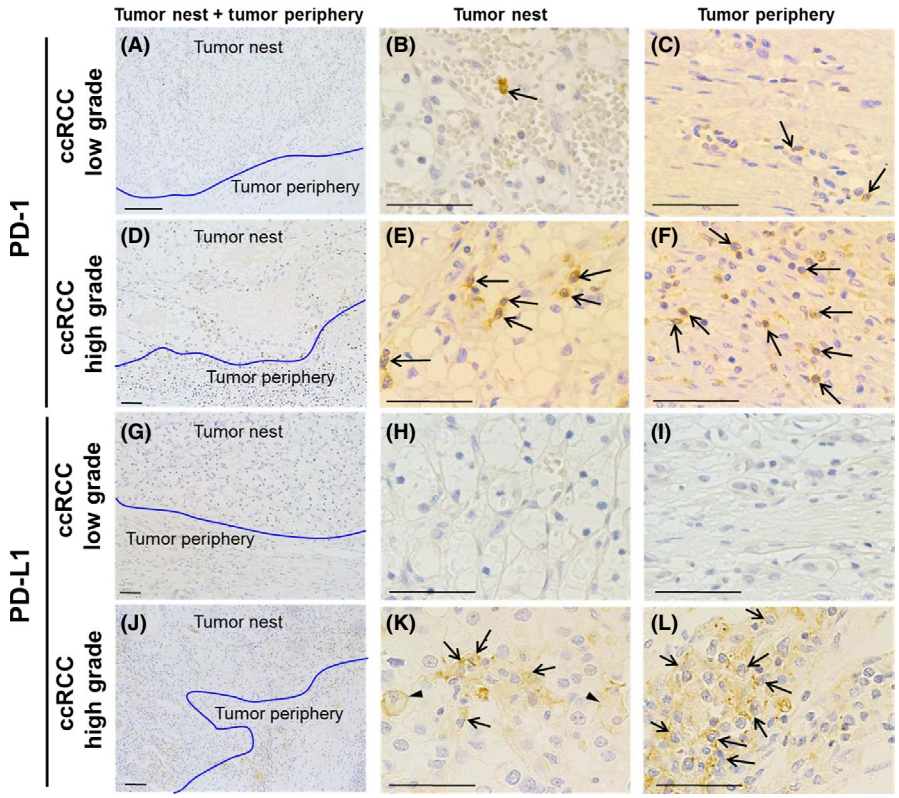

FIGURE 1 Immunohistochemical. expression of programmed death-1 (PD-1) and PD-ligand 1 (PD-L1) in low-grade and high-grade clear cell renal cell carcinoma (ccRCC) tissues without pretreatment. Paraffin sections were reacted with anti-PD-1 (A-F) or PD-L1 (G-L). In low-grade ccRCC, a small number of tumor-infiltrating immune cells (TIIC) were positive for PD-1 (A-C), whereas many PD-1-positive TIIC were found in high-grade tumors (D-F). No PD-L1 expression was found in low-grade ccRCC tissues (G-I), and PD-L1 expression was mainly observed in TIIC in high-grade tumors (J-L). Some tumor cells sporadically showed membranous PD-L1 staining (K). Scale line = 50 μm. Blue curve (A,D,G,J) outlines the areas of the tumor nest and periphery. Arrows in B, C, E, F indicate PD-1-positive TIIC, arrows in K, L indicate PD-L1-positive TIIC and arrowheads in K indicate PD-L1-positive tumor cells

PD-1-positive TIIC were observed at the tumor nest and tumor periphery (Fig. 1A-C, arrows), whereas many TIIC were observed in high-grade ccRCC tissues (Fig. 1D-F, arrows). Staining of PD-1 on TIIC was observed in 43 ccRCC cases (43%) at the tumor nest, whereas it was observed in 44 cases (44%) at the tumor periphery. Tumor cell expression of PD-1 was not observed. The mean PD-1-positive TIIC score at the tumor periphery was significantly higher than that at the tumor nest (8.2 vs 4.1) ($P < 0.001$). The correlation between PD-1 and PD-L1 expression in ccRCC and the clinicopathological parameters is summarized in Table 1. Programmed death-1 expression both at the tumor nest and tumor periphery was associated with pathological tumor stage, distant metastasis, and histological grade (Table 1).

In many low-grade ccRCC, neither PD-L1-positive TIIC nor PD-L1-positive tumor cells were observed (Fig. 1G-I). High-grade ccRCC often had PD-L1-positive TIIC at both the tumor nest and tumor periphery (Fig. 1J-L). Programmed death ligand-1-positive TIIC were found in 24 ccRCC cases (24%) at the tumor nest and 31 cases (31%) at the tumor periphery. The mean PD-L1-positive TIIC score at the tumor periphery was 2.7, and it was higher than that at the tumor nest (0.6) ($P = 0.011$). The PD-L1-positive TIIC score both at the tumor nest and tumor periphery was associated with pathological tumor stage and histological grade (Table 1). Programmed death ligand-1-positive tumor cells were observed in 18% of ccRCC, and the mean PD-L1-positive tumor score was 3.1. The PD-L1-positive tumor score was only associated with histological grade, but not with primary tumor stage, or regional or distant metastasis (Table 1).

3.2 | Prognostic significance of PD-1 and PD-L1 in patients with ccRCC without pretreatment

Receiver operating characteristic curve analysis was carried out to determine reasonable cut-off points for the PD-1-positive TIIC score, PD-L1-positive TIIC score, and PD-L1 tumor score (data now shown). Patients with ccRCC harboring a high PD-1-positive TIIC score at the tumor nest had significantly shorter progression-free and overall survival rates than those with tumors with a low PD-1-positive TIIC-positive score ($P = 0.007$ and $P < 0.001$) (Fig. 2A,B). Similarly, a high PD-1-positive TIIC score at the tumor periphery was associated with poor progression-free survival ($P = 0.006$) (Fig. 2C). No significant association was observed between the PD-1-positive TIIC score at the tumor periphery and overall survival ($P = 0.228$) (Fig. 2D).

The PD-L1-positive TIIC score at the tumor nest was not associated with progression-free survival ($P = 0.260$) (Fig. 2E), but patients with a high PD-L1-positive TIIC score at tumor nest had a significantly shorter overall survival than those with tumors harboring low PD-L1-positive TIIC score ($P < 0.001$) (Fig. 2F). A high PD-L1-positive TIIC score at the tumor periphery was not associated with progression-free survival ($P = 0.100$) (Fig. 2G), but it was associated with poor overall survival ($P < 0.001$) (Fig. 2H). Patients with ccRCC harboring a high PD-L1-positive tumor score had a slightly shorter progression-free survival and overall survival than those with a low PD-L1-positive tumor score ($P = 0.015$ and $P = 0.007$, respectively) (Fig. 2I,J).

TABLE 1 Association between programmed death-1 (PD-1) and PD-ligand 1 (PD-L1) expression and clinicopathological parameters in primary clear cell renal cell carcinoma without pretreatment

	Total number of cases	PD-1(+) TIIC tumor nest, mean ± SD	PD-1(+) TIIC tumor periphery, mean ± SD	PD-L1(+) TIIC tumor nest, mean ± SD	PD-L1(+) TIIC tumor periphery, mean ± SD	PD-L1(+) tumor cell (%), mean ± SD
Total	100	4.1 ± 7.0	8.2 ± 13.4	0.6 ± 2.6	2.7 ± 7.5	3.1 ± 12.0
Primary tumor stage						
pT1 + pT2	71	2.2 ± 4.5	5.1 ± 9.7	0.3 ± 0.7	1.7 ± 4.7	2.0 ± 8.7
pT3 + pT4	29	8.8 ± 9.6	15.8 ± 17.7	1.6 ± 4.7	5.0 ± 11.7	6.0 ± 17.6
P		<0.001	<0.001	0.024	0.049	0.130
Lymph node metastasis						
pN0	96	4.1 ± 7.0	8.3 ± 13.5	0.7 ± 2.7	2.8 ± 7.7	3.3 ± 12.3
pN1	4	4.5 ± 7.7	6.3 ± 11.2	0.0 ± 0.0	0.8 ± 1.5	0.0 ± 0.0
P		0.913	0.768	0.629	0.603	0.597
Distant metastasis						
pM0	91	3.3 ± 5.8	6.9 ± 11.8	0.7 ± 2.8	2.9 ± 7.9	2.9 ± 12.2
pM1	9	12.9 ± 11.9	21.8 ± 20.8	0.2 ± 0.7	0.6 ± 1.7	5.1 ± 11.0
P		<0.001	0.001	0.630	0.378	0.609
Histological grade						
G1 + G2	70	2.3 ± 4.8	4.9 ± 9.9	0.2 ± 0.6	1.2 ± 3.8	1.5 ± 8.3
G3 + G4	30	8.3 ± 9.4	16.0 ± 17.1	1.6 ± 4.7	6.0 ± 12.0	7.0 ± 17.6
P		<0.001	<0.001	0.015	0.003	0.034

3.3 | Clinical course of ccRCC patients without pretreatment and response to VEGF-TKIs for the treatment of metastatic disease

During the follow-up periods of 100 untreated primary ccRCC patients, 34 patients developed metastatic disease and 25 patients died of the disease. For the treatment of metastatic disease, 4 patients were treated with sorafenib and 14 patients were treated with sunitinib according to their respective protocols.[2,16] Six other patients were treated with interferon-α or interleukin-2, and metastases were removed in the other 4 patients. Among 18 patients treated with VEGF-TKIs (sorafenib or sunitinib), the treatment was ceased in 15 patients because of tumor progression or severe adverse effects, and 13 patients died of the disease during the follow-up period.

The objective response rate characterized by an experienced urologist (R.M.) according to RECIST was 5 patients (29%), all PR. Standard disease was noted as the best response for 10 patients (59%). The remaining 2 patients (12%) had PD as the best response. As shown in Fig. 3A-E, there was no significant association between the objective response and PD-1-positive TIIC score, PD-L1-positive TIIC score, or PD-L1-positive tumor score. In contrast, ccRCC tissues from the patients with PD after VEGF-TKI treatment had higher PD-1-positive TIIC scores at the tumor nest compared with tumors from those with PR or SD ($P = 0.045$) (Fig. 3F). A more clear correlation was observed between clinical benefit and PD-1 TIIC score at the tumor periphery ($P < 0.002$) (Fig. 3G). The PD-L1-positive TIIC scores at both the tumor nest and tumor periphery were also associated with clinical effects, and the difference in the mean PD-L1-positive TIIC score at the tumor periphery was greater than that at tumor nest ($P = 0.016$ vs $P < 0.001$) (Fig. 3H,I). There was no significant correlation between clinical effects and PD-L1 tumor score ($P = 0.934$) (Fig. 3J).

3.4 | Expression of PD-1 and PD-L1 in ccRCC tissues treated with VEGF-TKIs

Histologically, focal degeneration and necrosis were sporadically observed in primary ccRCC tissues treated with VEGF-TKIs, but viable tumor cells survived in all tumor tissues examined in this study. All primary ccRCC tissues treated with VEGF-TKIs (7 sorafenib-treated and 18 sunitinib-treated tumors) had PD-1-positive TIIC both at the tumor nest and tumor periphery (Fig. 4A-C), and there were many PD-1-positive TIIC in the primary ccRCC tissues treated with VEGF-TKIs (Fig. 4B,C, arrows). There were PD-L1-positive TIIC in 18 of 25 VEGF-TKI-treated ccRCC tissues both at the tumor nest and tumor periphery (72%) (Fig. 4E,F, arrows), and PD-L1-positive tumor cells were observed in 10 of 25 VEGF-TKI-treated tumors (40%) (Fig. 4D-F). The PD-L1-positive tumor cells were sporadically observed in ccRCC tissues (Fig. 4E, arrowheads).

The PD-1-positive TIIC scores at the tumor nest and tumor periphery were significantly higher in ccRCC tissues pretreated with VEGF-TKIs (sorafenib or sunitinib) than in untreated tumors ($P < 0.001$ and $P < 0.001$, respectively) (Fig. 5A,B). The PD-L1-positive TIIC scores at the tumor nest and tumor periphery were also significantly higher in VEGF-TKI-pretreated tumors (sorafenib or sunitinib) compared with those in untreated tumors ($P < 0.001$ and

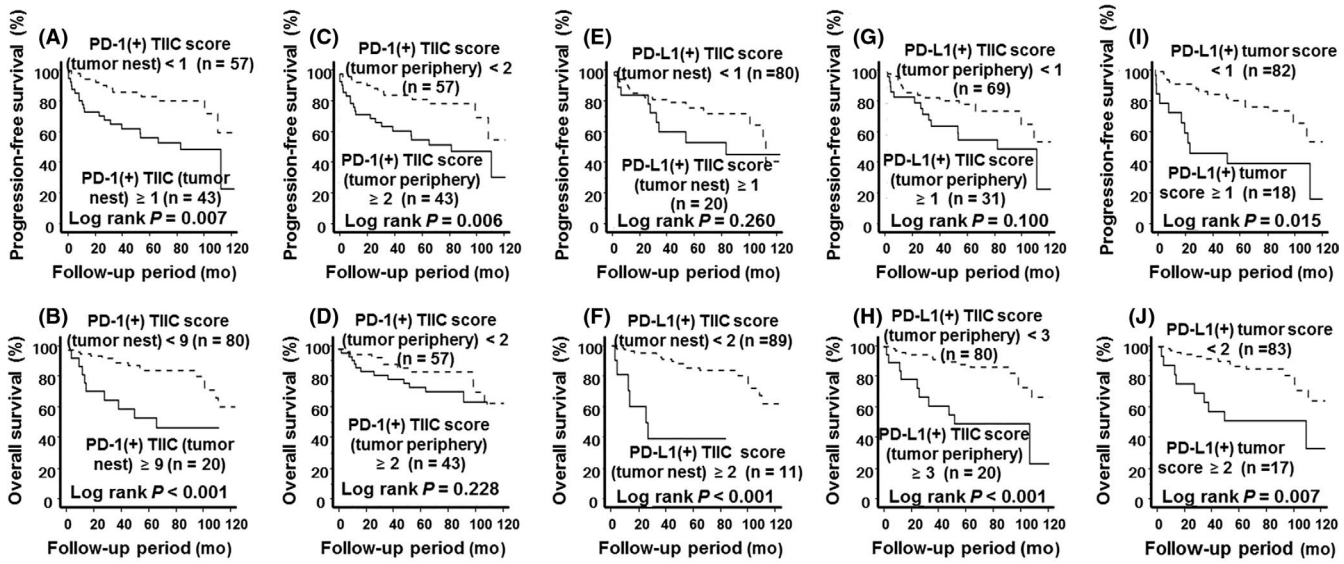

FIGURE 2 Kaplan-Meier curves of progression-free survival and overall survival according to programmed death-1 (PD-1)-positive tumor-infiltrating immune cell (TIIC) score at the tumor nest (A,B), PD-1-positive TIIC score at the tumor periphery (C,D), PD-ligand 1 (PD-L1)-positive TIIC score at the tumor nest (E,F), PD-ligand 1 (PD-L1)-positive TIIC score at the tumor periphery (G,H), and PD-L1 tumor score (I,J) in 100 untreated primary clear cell renal cell carcinomas. The cut-off points for the PD-1-positive TIIC score at the tumor nest for progression-free survival and overall survival were 1 and 9, and those at the tumor periphery were 2 and 2, respectively. The cut-off points for the PD-L1-positive TIIC score at the tumor nest for progression-free and overall survival were 1 and 2, and those at the tumor periphery were 1 and 3, respectively. The cut-off points for the PD-L1 tumor score were 1 and 2, respectively

$P < 0.001$, respectively) .(Fig. 5C,D). In contrast, there was no significant association between PD-L1 tumor score and VEGF-TKI treatment (Fig. 5E).

4 | DISCUSSION

In the present study, we found that both PD-1 and PD-L1 were predominantly expressed in the tumor microenvironment of high-grade ccRCC tissues, and that elevated expression of PD-1 and PD-L1 by TIIC was associated with poor prognosis, suggesting that the PD-1/PD-L1 pathway functions at the clinical level to impair immune surveillance, and thus foster tumor progression. Upregulated PD-1 and PD-L1 expression by TIIC was also associated with clinical effects in ccRCC patients treated with VEGF-TKIs for metastatic disease. Furthermore, we retrospectively evaluated the impact of PD-1 and PD-L1 expression in ccRCC tissues with VEGF-TKI pretreatment and found that VEGF-TKI-treated ccRCC tissues had significantly more PD-1-positive TIIC and PD-L1-positive TIIC in the tumor microenvironment than untreated tumors. These data further showed that overexpression of PD-1 and PD-L1 by TIIC is related to resistance to VEGF-TKI treatment, and that binding of PD-L1-positive TIIC to PD-1-positive TIIC might protect tumor cells from T cell responses against tumor cells. As responses to anti-PD-L1 Ab were mainly observed in patients with tumors expressing high levels of PD-L1, especially when PD-L1 was expressed by TIIC,[10] immunotherapy blocking the PD-1/PD-L1 axis could be effective for patients treated with VEGF-TKIs, especially for those with ccRCC with high PD-1/PD-L1-positive TIIC scores. Indeed, recent clinical trials of combined therapy using PD-1/PD-L1 axis blockade and VEGF-targeted therapy reported positive antitumor activity.[20] The poor prognosis and resistance to VEGF-TKI treatment in patients with high PD-1 and PD-L1 tumors in the present study provide a rationale for sequential treatment with PD-1/PD-L1 blockade after VEGF-targeted therapy.

Clear cell RCC has typical vessel formation and characteristic molecular background, with inactivation of the *VHL* gene and upregulation of hypoxia-inducible factor.[21] Hypoxia-inducible factor enhances the expression of proangiogenic factors such as VEGF and platelet-derived growth factor. Although VEGF is an important inducer of angiogenesis, there is accumulating evidence that VEGF

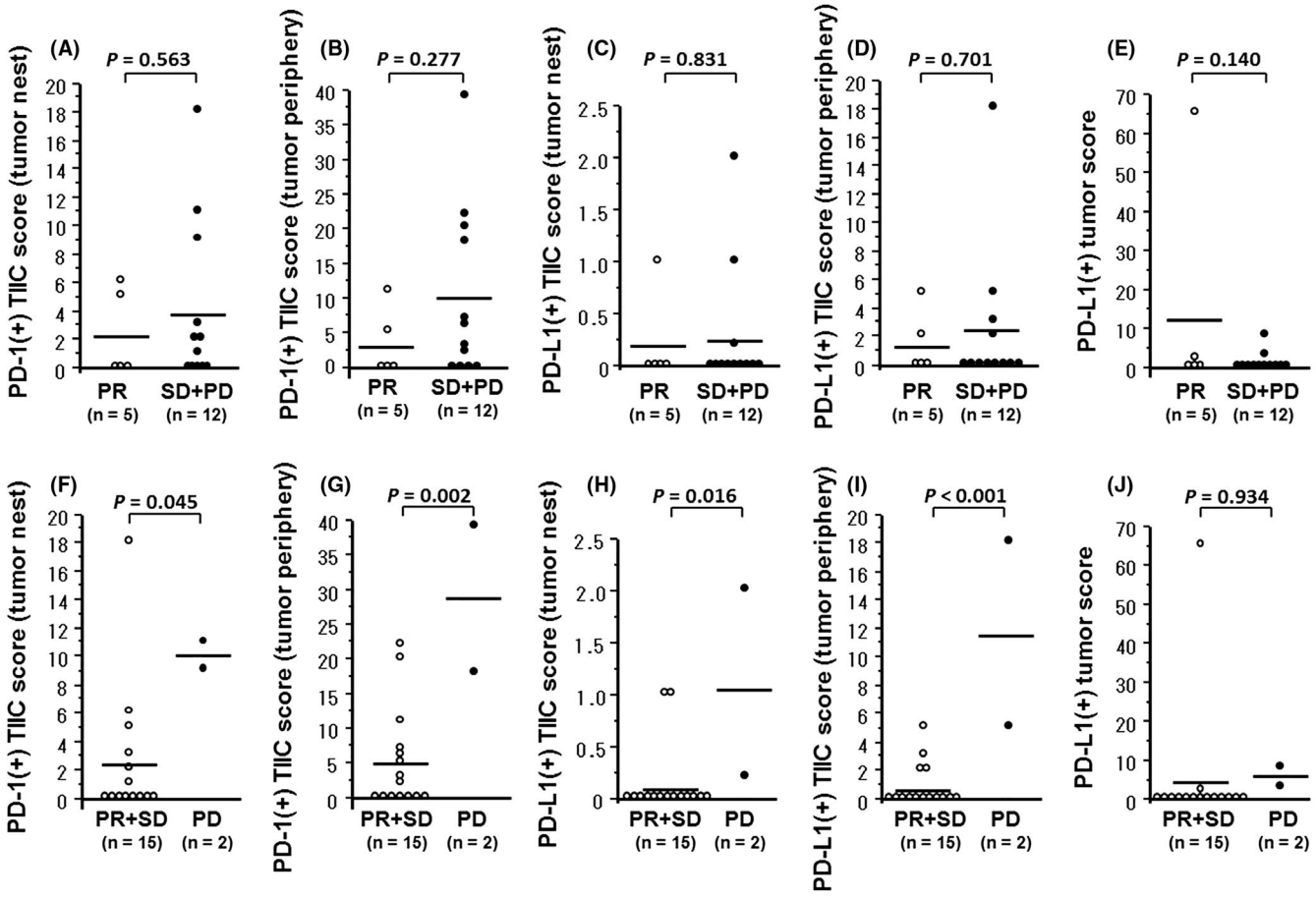

FIGURE 3 Correlation of programmed death (PD)-1-positive tumor-infiltrating immune cells (TIIC) score at the tumor nest and tumor periphery, PD-1 ligand (PD-L1)-positive TIIC score at the tumor nest and tumor periphery, or PD-L1-positive tumor score with objective response (partial response [PR] vs stable disease [SD] or progressive disease [PD]) (A-E) or clinical effects (PR or SD vs PR) (F-J) in clear cell renal cell carcinoma patients treated with vascular endothelial growth factor-tyrosine kinase inhibitors (VEGF-TKIs) for metastatic disease. Note that the PD-1-positive TIIC score and PD-L1-positive TIIC score were associated with clinical effects of VEGF-TKI treatment, whereas the PD-L1-positive tumor score was not

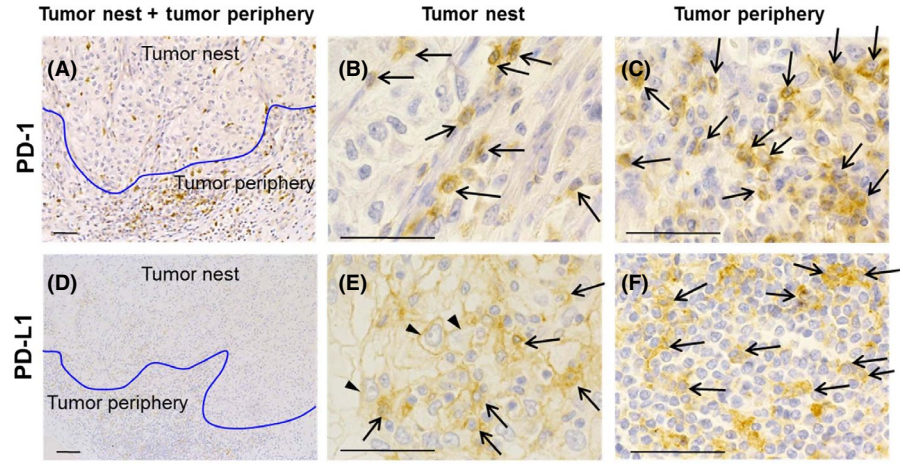

FIGURE 4 Immunohistochemical. expression of programmed-death (PD)-1 (A-C) and PD-ligand 1 (PD-L1) (D-F) in clear cell renal cell carcinoma treated with vascular endothelial growth factor-tyrosine kinase inhibitors. Scale line = 50 μm. Blue curve (A,D) outlines the areas of the tumor nest and periphery. Arrows in B, C indicate PD-1-positive TIIC, arrows in E, F indicate PD-L1-positive TIIC and arrowheads in E indicate PD-L1-positive tumor cells

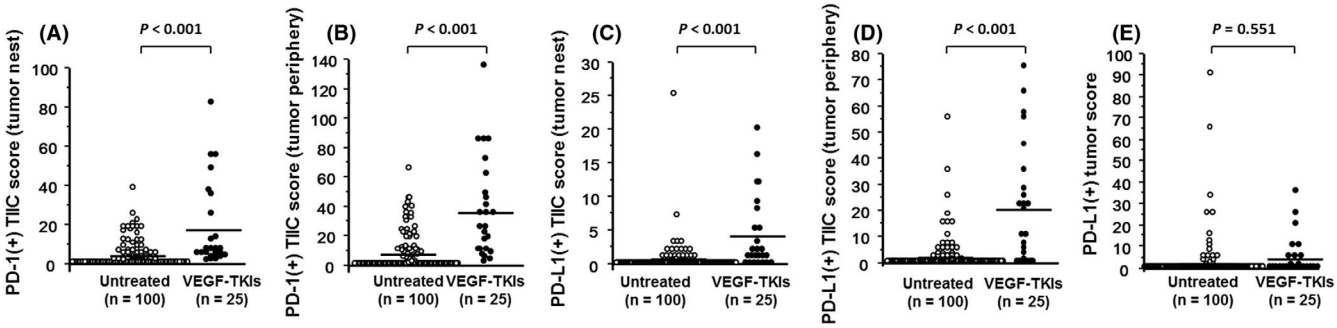

FIGURE 5 Statistical analysis of the differences in programmed death (PD)-1-positive tumor-infiltrating immune cell (TIIC) score at the tumor nest (A) and the tumor periphery (B), and PD-ligand (PD-L1) at tumor nest (C), tumor periphery (D) and PD-L1 tumor score (E) in clear cell renal cell carcinoma tissues with or without VEGF-TKI vascular endothelial growth factor-tyrosine kinase inhibitor treatment. Bars: indicate mean

also has immunosuppressive effects.[22] Therefore, ccRCC is an immunogenic tumor in which angiogenesis and immunosuppression work hand in hand, and its growth is associated with impaired tumor immunity. Moreover, ccRCC is an immunological tumor that is often abundant in TIIC,[23] and most patients with metastatic RCC receive immunotherapy with interferon-α or interleukin-2 as the standard therapy before the introduction of molecular-targeted therapy.[24] However, an elevated number of TIIC was associated with poor prognosis,[25,26] probably because increased T cell infiltration within ccRCC tissues is often impaired and incapable of mediating tumor rejection.[27] These findings suggest that ccRCC possesses a local mechanism to undermine antitumor immunity. In the current study, we found that both PD-1 and PD-L1 are expressed by TIIC within ccRCC tissues, and this is consistent with the notion that the PD-1/PD-L1 pathway might, at least in part, lead to the immunosuppression observed in patients with ccRCC. This suggests that blocking the PD-1/PD-L1 pathway can enhance anticancer immunity in ccRCCs, but little is known about the predictive factors of efficacy for therapy targeting PD-1/PD-L1 in ccRCC. Patients with ccRCC expressing high levels of PD-L1 by TIIC but not tumor cells, responded well to the anti-PD-L1 Ab,[10] suggesting that PD-1/PD-L1 expression by TIIC can be one predictive factor of treatment. As nivolumab, a novel immune checkpoint inhibitor, inhibits PD-1 not PD-L1,[28] it is necessary to investigate the association between PD-1 and PD-L1 expression by TIIC and the efficacy of PD-1/PD-L1 blockade in the future.

The success of PD-1/PD-L1 blockade therapies underlines the notion that tumor-specific T cell responses pre-exist in ccRCC patients and are controlled by immune modulatory mechanisms. T cells reactive to tumor-specific antigens (neoantigens) have been detected in many malignancies,[29] and neoantigens were found to be the target of checkpoint inhibitor-induced T cell responses.[30] Compared with other malignant solid tumors, RCC has more indel mutations, generating higher binding affinity neoantigens and more mutation-specific binders.[31] Furthermore, indel number is significantly associated with checkpoint inhibitor response in patients with melanoma.[31] Of note, integrative analyses of colorectal cancer revealed that a scoring system based on the quantification of cytotoxic and memory T cells in tumor tissues is a stronger predictor of colorectal cancer patients with microsatellite instability.[32] This study suggested that assessment of the immune status using immunohistochemistry is a strong

indicator of tumor recurrence beyond microsatellite instability, which could be an important guide for immunotherapy strategies. Furthermore, this study showed that PD-1 and PD-L1 expression by TIIC was associated with clinical effects in VEGF-TKI-treated patients, and that there are many PD-1-positive TIIC and PD-L1-positive TIIC in ccRCC tissues treated with VEGF-TKI. Taken together, these data suggest that VEGF-TKI-treated ccRCC highly infiltrated with PD-1-positive TIIC and PD-L1-positive TIIC will also benefit from immune checkpoint inhibitors, such as nivolumab, and these markers could be helpful for patient selection for immune therapy.

Within individual lesions, PD-L1 staining was heterogeneous and PD-L1-positive tumor cells were predominantly detected in high-grade areas.[33] As PD-L1 expression was mainly observed in high-grade tumor cells, whole slides were assessed to avoid false-negative results in the present study. In contrast to PD-L1 staining on tumor cells, to the best of our knowledge, the heterogeneity of the staining on TIIC was not reported. Therefore, PD-1 and PD-L1 staining on TIIC was evaluated at the tumor nest and tumor periphery, respectively. Both PD-1-positive and PD-L1-positive TIIC scores at the tumor nest and tumor periphery were associated with clinicopathological parameters and prognosis. Importantly, PD-1 and PD-L1 TIIC scores at the tumor periphery were more significantly correlated with the clinical effects in VEGF-TKI-treated ccRCC patients than the scores at the tumor nest. This suggested that the TIIC score at the tumor periphery reflects the immune status of the patients, and evaluation of both the tumor nest and tumor periphery is necessary to predict resistance to VEGF-TKI and likelihood of response to immunotherapy targeting the PD-1/PD-L1 pathway.

In conclusion, PD-1 and PD-L1 expression on TIIC were closely related to poor prognosis of ccRCC patients, and PD-1 and PD-L1 were also overexpressed on VEGF-TKI-treated ccRCC tissues. These findings revealed that PD-1 and PD-L1 expression on TIIC is potential antitumor biomarkers of sequential therapy by their inhibition after VEGF-TKI therapy.

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ETHICAL APPROVAL AND CONSENT TO PARTICIPATE

Patient tissue samples were collected between 1994 and 2014 at the Department of Urology of each participating institute. The study was approved by the ethics committee of each participating institute, and informed consent was obtained from all patients.

DATA AVAILABILITY

The datasets generated and/or analyzed during the current study are available from the corresponding author upon reasonable request.

DISCLOSURE

Ryuichi Mizuno: honoraria from Bristol-Myers Squibb, Novartis, Ono Pharmaceutical, Pfizer; Tsunenori Kondo: honoraria from Pfizer, Novartis, Ono Pharmaceutical, Bayer Yakuhin; Nobuo Shinohara: honoraria from Ono Pharmaceutical, Pfizer, Novartis, Chugai Parmaceutical, GSK, Janssen, MSD, Bayer Yakuhin, Takeda Pharmaceutical; grants from Ono Pharmaceutical, Astellas, Taiho Yakuhin; Norio Nonomura: honoraria from Astellas, Pfizer, Takeda Pharmaceutical; grants from Astellas, Ishihara Sangyo, Novartis, Takeda Pharmaceutical, Taiho Pharmaceutical, Sanofi, Nippon Kayaku, Nippon Shinyaku, Pfizer; Masatoshi Eto: honoraria from MSD, Ono Pharmaceutical, Chugai Pharmaceutical, Novartis, Pfizer, Bristol-Meyers Squibb; grants from Astellas, Kissei Pharmaceutical, Sanofi, Ono Pharmaceutical, Takeda Pharmaceutical; Hideyasu Matsuyama: honoraria from Janssen, MSD; grants from Janssen, MSD, Pfizer, Bayer Yakuhin, Baxter, Kyowa Hakko Kirin, Sanofi, Astellas, Takeda Pharmaceutical; Mototsugu Oya: honoraria from Pfizer, Novartis, Bayer Yakuhin, Ono Pharmaceutical, Bristol-Myers Squibb. The other authors declare no conflict of interest for this article.

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