

ORIGINAL RESEARCH



## Peripheral T cell receptor repertoire features predict durable responses to anti-PD-1 inhibitor monotherapy in advanced renal cell carcinoma

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### ABSTRACT

Immune checkpoint inhibitors (ICIs) offer significant clinical benefits to a subset of cancer patients via the induction of a systemic T cell-mediated anti-cancer immune response. Thus, the dynamic characterization of T cell repertoires in the peripheral blood has the potential to demonstrate noninvasive predictive biomarkers for the clinical efficacy of ICIs. In this study, we collected tumor tissues and peripheral blood samples from 25 patients with advanced kidney cancer before anti-programmed cell death protein 1 (PD-1) treatment and 1, 3, and 6 months after treatment initiation. Furthermore, we applied a next-generation sequencing approach to characterize T cell receptor (TCR) alpha and beta repertoires. TCR repertoire analysis revealed that the responders to anti-PD-1 showed an expansion of certain T cell clones even in the blood, as evidenced by the significant decrease in the TCR diversity index and increase in the number of expanded TCR clonotypes 1 month after treatment. Interestingly, these expanded TCR clonotypes in the peripheral blood were significantly shared with tumor-infiltrating T cells in responders, indicating that they have many circulating T cells that may recognize cancer antigens. Expression analysis also revealed that 1 month after treatment, T cells from the peripheral blood of responders showed significantly elevated transcriptional levels of *Granzyme B*, *Perforin*, *CD39*, and *PD-1*, markers of cancer-associated antigen-specific T cells. Altogether, we propose that global TCR repertoire analysis may allow identifying early surrogate biomarkers in the peripheral blood for predicting clinical responses to anti-PD-1 monotherapy.

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### KEYWORDS

Immune checkpoint inhibitors; predictive marker; T cell receptor; renal cell carcinoma; next-generation sequencing

## Introduction

Cancer immunotherapy has revolutionized the field of cancer in recent years and has remarkably improved the prognosis of cancer patients.<sup>1–3</sup> Particularly, the success of immune checkpoint inhibitors (ICIs) has highlighted the importance of an effective anti-cancer immune response in cancer patients. However, the response rates to ICIs are limited and the majority of patients still not benefit from the treatment with ICIs; in fact, some of them experience severe immune-related adverse events.<sup>4,5</sup> To date, various classes of biomarkers, such as the tumor mutation burden or the expression of programmed death-ligand 1 (PD-L1) in cancer tissues have been proposed to have a significant impact on the clinical response to ICIs.<sup>3,6,7</sup> However, these markers focus on the state of the tumor at the time of cancer diagnosis and sometimes produce contradictory outcomes since tumors are generally subjected to drug-imposed selective pressure before the initiation of ICIs. Moreover, repeated tumor biopsies are not feasible and may not be performed before ICI treatment. To overcome these limitations, it is urgent to pursue novel noninvasive biomarkers of responses to ICI that allow the “early” determination of clinical benefits.

T cell receptors (TCR), mainly consisting of a heterodimer of TCR alpha (TCRA) and beta (TCRB) chains are expressed on the surface of T cells.<sup>8,9</sup> To recognize a large variety of

antigens, TCR genes undergo somatic V-(D)-J recombination that generates a huge diversity of T cell repertoires. Using next-generation sequencing-based TCR analysis, several studies have attempted to determine whether TCR diversity in the peripheral blood can serve as a useful indicator of prognosis in various types of cancer.<sup>10–12</sup> In fact, recently, several studies reported that peripheral TCR diversity at baseline could be either a predictive or a prognostic biomarker for cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) or programmed cell death protein 1 (PD-1) inhibitors.<sup>13,14</sup> However, these conclusions oppose those reported in other study,<sup>15</sup> perhaps due to differences in the types and clinical stages of cancers investigated. Therefore, we hypothesized that the comparison of TCR diversity before and after ICI treatment could be used for the stratification of patients responding to ICIs in each type of cancer. Of note, especially in urological cancers, the dynamics of peripheral TCR repertoires after treatment with ICIs remains unknown.

In the present study, we indicated that the representative ICI, nivolumab (anti-PD-1 inhibitor), could induce strong immune reactions as evidenced by the expansion of abundant T cells even in the peripheral blood of responder metastatic renal cell carcinoma (mRCC) patients. Interestingly, responders sustained the significantly expanded T cell clonotypes in

the peripheral blood 1 month after initiating the anti-PD-1 therapy for a long time. These expanded T cells were also detected in pretreatment tumor tissues, indicating that responders have many circulating T cells that may recognize cancer-antigens. Furthermore, we found that the transcriptional levels of *CD39*, *PD-1*, and *GZMB*, known markers of cancer-associated antigen-specific T cells,<sup>16,17</sup> were significantly increased on the T cells from the peripheral blood of responders 1 month after the initiation of anti-PD-1.

In the field of mRCC, nivolumab emerged as a new target therapy for second- or further-line treatment based on the results of a clinical trial (checkmate 025).<sup>1</sup> Although the efficacy of nivolumab was also proven in patients with mRCC resistant to tyrosine kinase inhibitors, the objective response rate is around 25% in real-life settings.<sup>18–20</sup> Given the current situation, we believe that T cell repertoire analysis and immune-profiling of PBMCs in nivolumab-treated cancer patients at an early time point may concisely and practically predict clinical response and avoid unnecessary continued therapy.

## Materials and methods

### Study design

Between June 2017 and June 2019, a total of 25 kidney cancer patients were enrolled in this study and received the anti-PD-1 inhibitor nivolumab at 3 mg/kg every 2 weeks. All patients were diagnosed with RCC via nephrectomy or needle biopsy and had metastatic sites. Follow-up was performed according to the institutional standard for advanced RCC, with clinical examination and laboratory testing every 3 months and analysis by computed tomography (CT) scan every 3 months. The objective response rate (ORR) was evaluated using the Response Evaluation Criteria in Solid Tumors version 1.1,<sup>21</sup> and was defined as the proportion of patients achieving complete response (CR) and partial response (PR). Blood samples were collected from the 25 patients, before and 1, 3, and 6 months after anti-PD-1 treatment. Additionally, we also obtained pre-treatment tumor tissues from 15 patients (A2, A5, A6, A8, A13, A14, A15, A16, A18, A19, A21, A23, A26, A29, and A30).

The study protocol was approved by the Institutional Review Board of Osaka University (approval number 668-5). All patients provided written informed consent.

### T cell receptor sequencing

Total RNA from the tumor tissues and PBMCs was isolated using the RNeasy Mini kit (Qiagen, Valencia, CA, USA). Sequencing libraries of TCR were prepared as described previously<sup>10,22</sup> and subjected to sequencing on the Illumina Miseq platform, using the 600 cycles Miseq Reagent Kit V3 (Illumina, San Diego, CA USA). Sequencing data analysis was performed using Tcrp as described previously.<sup>23</sup> Briefly, sequencing reads were mapped to the TCR reference sequences obtained from IMGT/GENE-DB (<http://www.imgt.org>) using the Bowtie2 aligner (version 2.1.0); after decomposition of the sequencing reads into the V, (D), and J segments, CDR3 were searched. The inverse Simpson's diversity

index (DI) and the clonality based on the Shannon index in the context of CDR3 sequences were used to evaluate TCR clonality.<sup>14,24,25</sup> Of note, the DI increases when TCR clones are evenly distributed, and decreases with the enrichment of certain T cell clones; on the other hand, a perfectly monoclonal population and a maximally diverse population are associated with clonality scores of 1 and 0, respectively. The DIs before, and 1, 3, and 6 months after anti-PD-1 treatment are shown in Supplementary Table 1. A change in the DI was defined as DI (post-treatment)/DI (baseline).

### Gene expression analysis

cDNA was synthesized from total RNA using the Prime Script RT reagent kit (TAKARA BIO Inc., Shiga, Japan). Real-time RT-PCR was performed using the TaqMan gene expression assay (ThermoFisher, Waltham, MA, USA) in the QuantStudio 5 system (ThermoFisher), according to the manufacturer's instructions. All TaqMan probes used in this study are listed in Supplementary Table 2.

### Statistical analysis

The JMP Pro 14.0.0 software (SAS Institute Inc., Cary, NC) was used. The Mann-Whitney *U* test (two-tailed) was performed to detect significant differences between responders and non-responders in the context of DIs, and the of total number of expanded clones, 1,3, and 6 months after anti-PD-1 treatment. A paired *t*-test was used to compare the total proportion of the 10 most abundant TCR CDR3 clonotypes and the gene expression levels, pre- and post-anti-PD-1 treatment. To analyze the changes in CDR3 clonotypes in PBMCs, we counted expanded and contracted clones using the R package tcrSeqR. Briefly, we used CDR3 clonotypes with more than 6 reads in total in pre- and post-treatment samples, and applied the Fisher's exact test to examine the significant changes. Multiple testing was adjusted using the Benjamini-Hochberg method. Differences were considered significant at  $p < .05$ . The Kaplan–Meier method was used to calculate survival rates. The overall survival (OS) and progression-free survival (PFS) were defined as the interval from the start of anti-PD-1 treatment to death or disease progression, respectively. Log-rank tests were used for comparisons between the two groups.

## Results

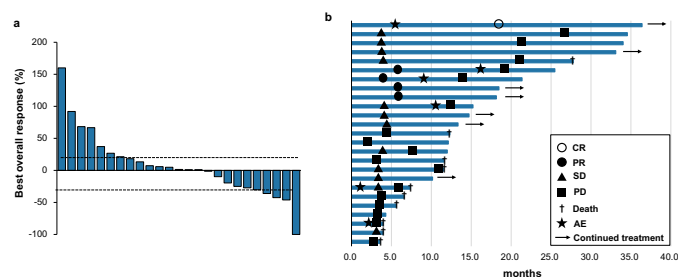
### Patients' characteristics

The clinical characteristics of all patients are summarized in Table 1. Twenty-two tumors were histologically diagnosed as clear-cell RCC. The median age of the patients was 65 years (range, 45–80 years). All patients received at least one prior treatment for metastatic cancer before receiving the anti-PD-1 therapy. The ORR was 20% (5 out of 25 patients, Table 1 and Figure 1(a)) At the time of analysis, twelve patients had sustained responses for 10 months or longer (Figure 1(b)). The median PFS and OS were 6.6 months (range, 2.5. 36.4 months) and 16.6 months (range, 5.7. 36.4 months), respectively. In the present study, considering the median PFS in the clinical trial of nivolumab

**Table 1.** Characteristics of the entire cohort.

Characteristics	
Median age, years (range)	65 (45–80)
Gender, n (%)	
Male	21 (84)
Female	4 (16)
Histopathology, n (%)	
Clear cell RCC	22 (88)
Papillary RCC	1 (4)
Unclassified	1 (4)
Collecting duct carcinoma	1 (4)
IMDC risk, n (%)	
Favorable	1 (4)
Intermediate	19 (76)
Poor	5 (20)
Nivolumab treatment line, n (%)	
2 <sup>nd</sup>	18 (72)
≥ 3 <sup>rd</sup>	7 (28)
Number of metastatic organs, n (%)	
Single	7 (28)
Multiple	18 (72)
Metastatic site	
Bone	10 (28)
Lung	15 (28)
Liver	6 (28)
Lymph nodes	6 (28)
Other	9 (28)
Best objective response, n (%)	
CR	1 (4)
PR	4 (16)
SD ≥ 6M	11 (44)
SD < 6M	2 (8)
PD	7 (28)
ORR	5 (20)
CBR	16 (64)

CBR, clinical benefit rate; CR, complete response; ORR, objective response rate; PD, progressive disease; PR, partial response. RCC, renal cell carcinoma; SD, stable disease.



**Figure 1.** Clinical efficacy in patients treated with nivolumab monotherapy. Response to nivolumab: (a) waterfall plot and (b) swimmers plot. Patients with continued nivolumab monotherapy beyond the data cutoff date are identified with arrows. The upper and lower dotted lines indicate 20% and –30% of the overall response rate. AE, adverse event; CR, complete response; PD, progressive disease; PR, partial response. SD; stable disease.

(Checkmate 025),<sup>1</sup> we defined responders as patients with CR, PR, or stable disease (SD) for ≥ 6 months (long SD). As a result, responders and non-responders consisted of 15 and 10 cases, respectively. Of note, no grade 4 or 5 treatment-related adverse events were observed (Supplementary Table 3).

### The diversity index significantly decreased early after treatment initiation concerning the PBMCs of the responders

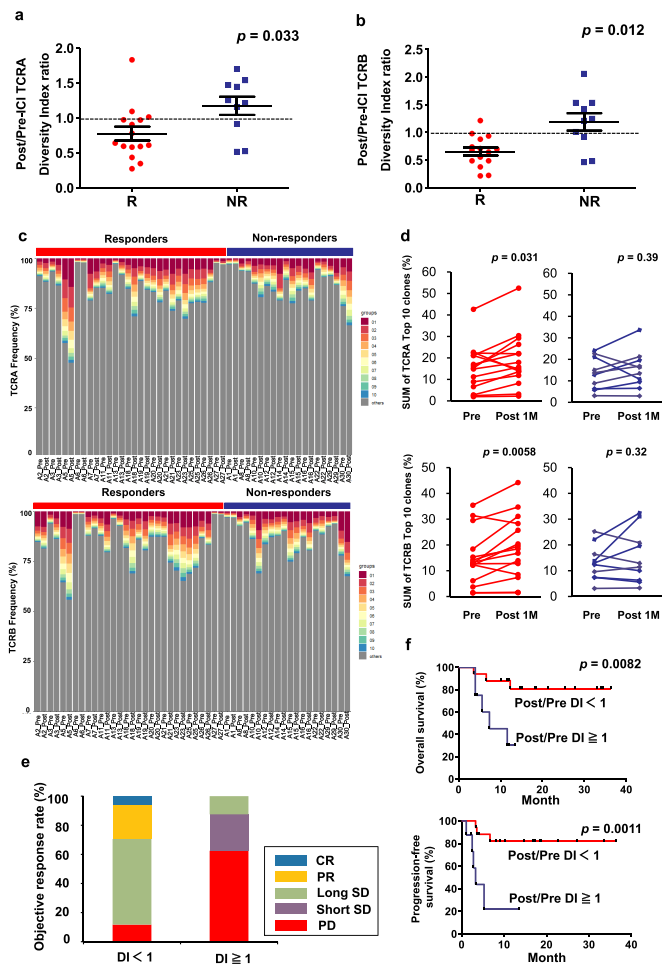
To characterize the changes in the systemic activation of T cells, we performed TCR sequencing on PBMCs obtained from 25

patients before and after the initiation of anti-PD-1 treatment. We first measured TCR diversity via the calculation of the DIs; of note, the DIs for TCRA and TCRB before anti-PD-1 therapy could not predict the clinical response (data not shown). We further investigated the changes in the DIs with respect to PBMCs after the introduction of anti-PD-1 therapy and found that the DIs for TCRA and TCRB were significantly decreased 1 month after treatment in responders *versus* non-responders ( $p = .033$  and  $p = .012$ , respectively, Figure 2(a,b)), and tended to decrease 2 months later ( $p = .071$  and  $p = .032$ , respectively, Supplementary Figure 1). Of note, no significant correlation was found between the DIs for TCR and the absolute lymphocyte counts at baseline. Moreover, the absolute lymphocyte counts 1 month after treatment did not change when compared to 3 and 6 months after treatment (data not shown), excluding the possibility that the TCR repertoire would reflect lymphocyte counts. Next, after sorting out TCRA and TCRB CDR3 clonotypes according to their frequencies in PBMC samples, we counted the proportions of abundant CDR3 clonotypes and found that the sum of the 10 most abundant CDR3 clonotypes with respect to both TCRA and TCRB was significantly increased in responders (Figure 2(c,d), Supplementary Table 4).

Interestingly, CR, PR, and long SD accounted for 83.3% of patients with a decrease in the DI (DI < 1 group), whereas all patients with an increase in the DI (DI ≥ 1 group) were associated with short SD and progressive disease (PD) (Figure 2(e)). Importantly the PFS and OS after the first administration of anti-PD-1 were significantly longer in the DI < 1 group than in the DI ≥ 1 group ( $p = .0011$  and  $p = .0082$ , respectively, Figure 2(f)), whereas the median DI 1, 3, and 6 months after treatment initiation had no influence on the clinical outcome (Supplementary Figure 2). Moreover, we measured TCR clonality as an alternative index of TCR diversity and evaluated the correlation between patient prognosis and TCR clonality (Supplementary Figure 3). Patients with increased TCR clonality indicated a superior OS and PFS compared with those with decreased TCR clonality ( $p = .044$  and  $p = .028$ , respectively). On the other hand, the change in the levels of inflammatory factors (considered useful prognostic markers for RCC patients), including (serum) C-reactive protein (CRP) before and after anti-PD-1 therapy, did not correlate with the clinical prognosis (Supplementary Figure 4). Taken together, our results suggest that a change in the TCR repertoire early after treatment initiation is a more informative and feasible therapeutic biomarker for patients undergoing anti-PD-1 therapy, compared with the static biomarkers assessed at baseline.

### The clonal T cell expansion in responders sustained long-term anti-cancer immune responses

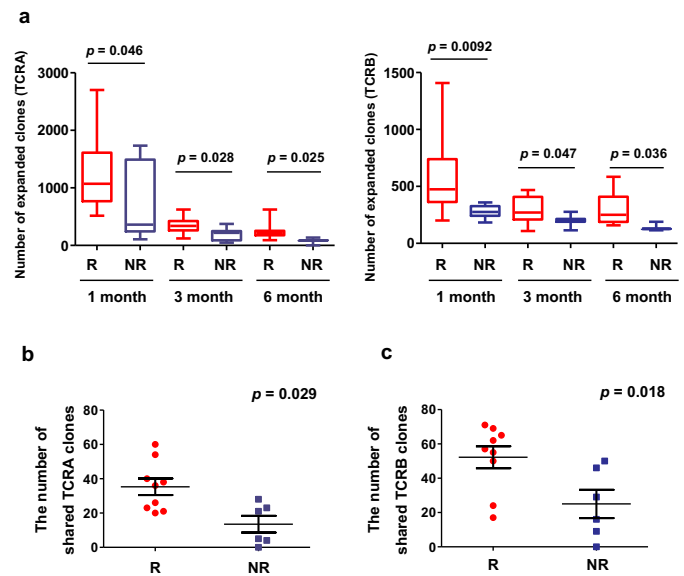
To better understand TCR repertoire dynamics associated with clinical response, we counted the number of TCRA and TCRB clones that significantly expanded (defined by a frequency of ≥ 0.01%) 1 month after treatment initiation. The majority of expanded clones were derived from novel clonotypes that had not been previously observed in the blood at baseline; however, there was no significant difference between responders and non-responders (Supplementary Figure 5). In contrast, when we measured the sum of novel and preexisting peripheral T cell clones at



**Figure 2.** Clonal T cell expansion in the peripheral blood of nivolumab responders. (a, b) Responders showed significantly decreased T cell receptor alpha (TCRA) and beta (TCRB) diversity indexes (DIs) in the peripheral blood 1 month after treatment initiation (*versus* non-responders). R; Responders, NR; Non-responders. The dotted lines refer to DIs equal at baseline and 1 month after treatment. (c) Bar plot showing the distribution of the 10 most abundant TCRA and TCRB CDR3 clonotypes in the peripheral blood. The gray color refers to CDR3 clonotypes other than the 10 most frequent. (d) The total proportion of the 10 most abundant TCRA and TCRB CDR3 clonotypes in the peripheral blood was significantly increased in responders *versus* non-responders. ( $p = .0031$  and  $p = .0058$  for TCRA and TCRB, respectively). The red circles and blue squares represent PBMC samples from responders and non-responders, respectively. (e) Objective response rate during nivolumab treatment according to the change of the TCRB diversity index 1 month after treatment initiation. CR; complete response, PR; partial response, PD; progressive disease, SD; stable disease. (f) Overall survival and progression-free survival after nivolumab initiation according to the change of the TCRB diversity index 1 month after treatment initiation. Differences between the two groups were assessed using the log-rank test.

baseline, responders exhibited a significantly higher number of expanded clones than those of non-responders, 1 month after treatment ( $p = .046$  and  $p = .0092$  for TCRA and TCRB, **Figure 3(a)**). Importantly, these expanded clones were significantly sustained, 3 and 6 months after treatment initiation in responders, suggesting long-lasting anti-cancer immune responses ( $p = .047$ ,  $p = .036$ , **Figure 3(a)**).

Accumulating evidence has suggested that tumor-infiltrating T cells are composed of cells able to recognize cancer-specific antigens (neoantigens and shared antigens).<sup>26,27</sup> We further examined TCR CDR3 clonotypes in the context of pre-treatment tumor tissues, and found that



**Figure 3.** The dynamics of the peripheral expanded T cell repertoire after the initiation of nivolumab. (a) Responders exhibited a higher number of expanded T cell clones 1 month after treatment initiation (frequency of  $\geq 0.01\%$ ) which was preserved over time. R; Responders, NR; Non-responders. (b, c) The number of peripheral T cell clones 1 month after treatment initiation shared with tumor-infiltrating T cells in pre-treatment tumor samples (regarding the 100 most abundant T cell receptor alpha (TCRA) and beta (TCRB) clonotypes). Responders showed more shared T cell clones than non-responders.

responders had significantly higher numbers of peripheral T cell clones shared with tumor-infiltrating T cells than those in non-responders 1 month after anti-PD-1 initiation (as per the 100 most abundant T cell clonotypes in the peripheral blood;  $p = .039$ ,  $p = .0018$ , **Figure 3(b,c)**); of note, the number of peripheral T cell clones shared with tumor-infiltrating T cells before treatment initiation was not significantly different between responders and non-responders (**Supplementary Figure 6**). These findings may indicate that tumor-infiltrating T cells circulate in the peripheral blood in responding to anti-PD-1, reflecting systemic anti-cancer immune responses.

### Immune-related gene expression early after the initiation of anti-PD-1

To characterize the changes in the systemic immune signature early after the initiation of anti-PD-1, we examined the transcriptional levels of immune-related genes associated with cytotoxic, effector, and exhausted T cells within PBMCs using real-time RT-PCR (**Figure 4(a-c)**). In the PBMCs, 1 month after the initiation of anti-PD-1, we found a significant increase in the ratio of Granzyme B (*GZMB*)/*CD3* and perforin (*PRF1*)/*CD3* in responder *versus* non-responder patients, implying that cytolytic T cells were strongly expanded by the anti-PD-1 inhibitor in responders ( $p = 0.0039$  and  $p = 0.0033$ , **Figure 4(b)**). Interestingly, the ratios of *CD39*/*CD8* and *PD-1*/*CD8* were also significantly increased in responders 1 month after the initiation of anti-PD-1 (**Figure 4(b)**), suggesting the systemic activation of  $CD8^+CD39^+$  and  $CD8^+PD-1^+$  T cells, closely related to cancer antigen-specific T cells.<sup>16,17</sup>

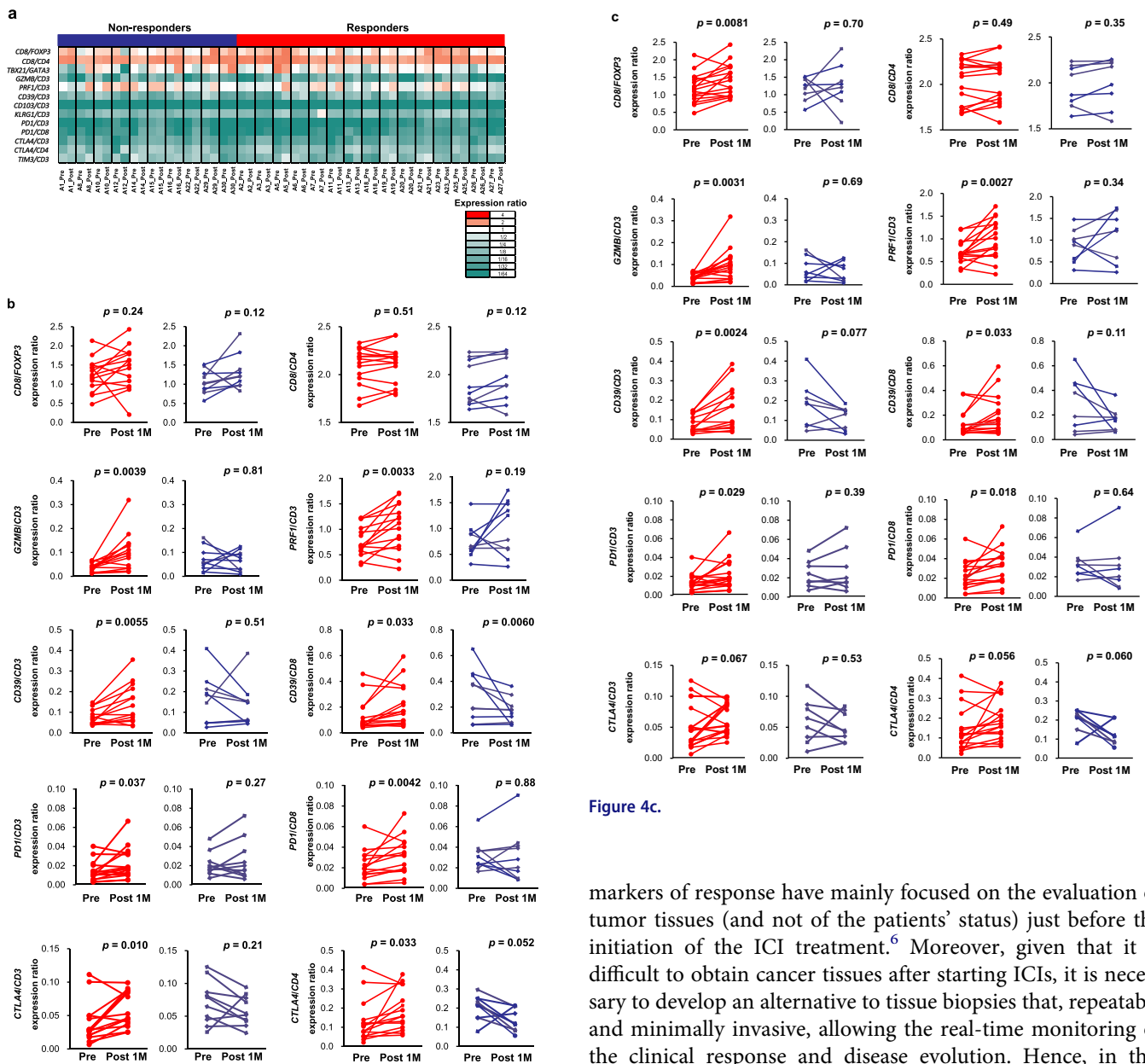


Figure 4c.

**Figure 4.** Expression of immune-related genes in blood samples before and after the initiation of nivolumab. (a) The heatmap shows the expression ratios of 14 immune-related genes in PBMCs collected before (pre) and 1 month after the initiation of nivolumab. (b, c) Line graphs show the comparative analysis of the expression ratios of immune-related genes in pre-treatment (pre) and post-treatment (post 1 M) PBMCs. Red circles and blue squares represent PBMC samples from responders and non-responders (b) or from the  $DI < 1$  group and the  $DI \geq 1$  group (c), respectively. The y-axis refers to the expression ratio of each gene.  $p$  values were calculated using the paired  $t$  test.

## Discussion

Immunotherapy targeting immune checkpoints is now the most attractive therapy in the cancer field and has improved the prognosis of cancer patients.<sup>5,28,29</sup> However, the response rate of ICI monotherapy ranges from 11% to 40%, and treatment-related toxicity often causes discontinuation of ICIs.<sup>30</sup> Therefore, it is crucial to identify biomarkers to predict the response of patients to the immunotherapeutic strategies to enable a precision medicine approach. To date, predictive

markers of response have mainly focused on the evaluation of tumor tissues (and not of the patients' status) just before the initiation of the ICI treatment.<sup>6</sup> Moreover, given that it is difficult to obtain cancer tissues after starting ICIs, it is necessary to develop an alternative to tissue biopsies that, repeatable and minimally invasive, allowing the real-time monitoring of the clinical response and disease evolution. Hence, in this study, we performed TCR repertoire profiling combined with expression analysis of immune-related genes in PBMCs from patients treated with anti-PD-1 monotherapy. We aimed to identify "early" predictive markers of clinical response and to explore the mechanisms of action of ICIs.

First, the analysis of the peripheral blood T cell repertoire revealed a significant expansion of T cell clones (*versus* the baseline), early after treatment initiation in responders to nivolumab monotherapy, as evidenced by the decrease in the TCR DIs (Figure 2(a,b)). Our findings partially align with those reported by Han and colleagues that described the increased clonality of peripheral blood  $CD8^+PD-1^+$  T cells after 4–6 weeks of treatment with anti-PD-1/PD-L1; of note, this correlated with a superior PFS and OS compared with those in the context of decreased TCR clonality in non-small cell lung cancer patients.<sup>14</sup> On the other hand, a previous study on the change in TCR diversity could not stratify the response to anti-PD-1 therapy in pancreatic cancer.<sup>15</sup> We speculate that this difference is due to the different treatment regimen used (combination therapy with anti-PD-1, GVAX, mesothelin-

expressing *Listeria monocytogenes*, and cyclophosphamide) and the low mutation burden rate in pancreatic cancer, which lead a lower chance of the induction of cancer-antigen specific T cells.<sup>31</sup> Given that mRCC patients normally receive anti-PD-1 monotherapy, our results may clearly reflect the early changes in TCR diversity, which are associated with the clinical response.

Second, we found that the dynamics of the peripheral T cell repertoire was also different in nivolumab responders *versus* non-responders; the former exhibited a greater number of expanded T cell clones early (1 month) after the initiation of nivolumab, that was sustained over time (Figure 3(a)). Our results align with the data published by Hopkins *et al.* reported that long-term survivors (patients with  $\geq 6$  months OS) receiving anti-CTLA-4-based therapies exhibited more expanded T cell clones 3 months after treatment initiation when compared with short-term survivors (patients with  $< 6$  months OS) in the context of metastatic pancreatic cancer patients.<sup>15</sup> Moreover, in the present study, anti-PD-1 monotherapy also induced the expansion of T cell clones at a very early phase post-treatment in responders (Figure 3(a)). Again, this goes in line with a previously published paper reporting that the total number of expanding clones in the peripheral blood at day 21 was associated with the oncological outcome 6 months after anti-PD-1 treatment in metastatic melanoma patients.<sup>17</sup> Interestingly, 57% of these expanded T cell clones (range, 17–71% per the 100 most abundant T cell clonotypes) were also detected in the pre-treatment tumor samples (Figure 3(b, c)), indicating that clonally-expanded T cells in tumors, possibly recognizing cancer cells, can circulate systemically in responders. Recently, several studies reported that CTLA-4 blockade may increase the number of unique TCR clonotypes and induce peripheral T cell turnover after only one treatment cycle.<sup>32,33</sup> Given the different targets of anti-PD-1 and anti-CTLA-4 antibodies,<sup>34</sup> these findings suggest that the combination of anti-PD-1 and anti-CTLA-4 therapies may improve the chances of induction of a larger number of expanded T cell clonotypes and trigger the cancer-immunity cycle; of note, such a combination is sustainable.<sup>34</sup>

Third, we found that the expression levels of the T cells cytolytic markers *GZMB*, and *PRF1* were significantly increased in the peripheral blood 1 month after the initiation of anti-PD-1, indicating that the cytolytic activity of cytotoxic T lymphocytes (CTLs) was restored via the blockade of the PD-1 pathway. Another important finding was the significant increase in the *CD39/CD8* and *PD-1/CD8* ratios in responders, suggesting that anti-PD-1 may significantly increase the number of CD8<sup>+</sup>CD39<sup>+</sup> and CD8<sup>+</sup>PD1<sup>+</sup> T cells, thought to recognize cancer-associated antigens,<sup>16,17,35</sup> in the peripheral blood of responders. These results align with those in a study showing that the proliferative response of peripheral blood CD8<sup>+</sup>PD-1<sup>+</sup> T cells after the first week of treatment correlated significantly with improved prognoses in the context of anti-PD-1 therapy.<sup>36</sup> Altogether, these results indicate that anti-PD-1 may induce the expansion of CTLs recognizing cancer antigen-specific T cells in responders; therefore, further analyses of peripheral TCR repertoires using sorted CD8+PD-1<sup>+</sup> T cells may reveal more precise predictive markers in mRCC patients.

There are some limitations in this study. First, our small sample size may limit the generalization of our results to other types of cancer. Further investigations are needed to validate our results in a larger number of patients, ideally in the form of multi-institutional studies. Second, further studies are needed to deeply investigate the transcriptional levels of T cell subsets (CD8 and CD4) using sorted populations. Finally, complementary studies using additional cohorts are needed to clearly understand if expanded T cell clones in responders can recognize cancer-specific antigens such as shared antigens or neoantigens.

In conclusion, to our knowledge, this is the first study showing a peripheral predictive biomarker of the clinical response to anti-PD-1 monotherapy in urological cancers. Our data indicate that both the TCR dynamics and the expression levels of *GZMB*, *PRF1*, *CD39*, and *PD-1* in the peripheral blood may predict the response to anti-PD-1 early after treatment initiation. These surrogate biomarkers may support the discontinuation of anti-PD-1 in non-responders, allowing the physicians to select the next line of therapy in mRCC patients.

## Abbreviations

CDR3	complementarity-determining region 3
ICIs	immune checkpoint inhibitors
PBMCs	peripheral blood mononuclear cells
PD-1	programmed cell death protein 1
PD-L1	programmed death-ligand 1
TCR	T cell receptor
TCRA	T cell receptor alpha
TCRB	T cell receptor beta.

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## Authors' contribution

T.K. planned the entire project, performed data analysis, and drafted the manuscript. K.K. designed the study, conducted the experiments, performed data analysis, and revised the article. M.U. planned and supervised the entire project, and revised the article. N.N. was behind the study design and the working hypothesis and revised the article. E.T., Y.K., M.M., Y.H., K.N., Y.I., C.W., K.H., A.K., T.U., and K.F. performed data analysis and provided scientific advice.

## Disclosure of potential conflicts of interest

The authors declare no potential conflicts of interest.

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