의학박사 학위논문

Exploration of tumor microenvironment using multiplexed immunohistochemistry and its association with response in advanced or metastatic renal cell carcinoma treated with immune checkpoint inhibitors

면역관문억제제 치료한 신장암 환자에서 다중 면역조직화학검사를 이용한 종양미세환경 탐색 및 치료 반응 연관성 분석

울 산 대 학 교 대 학 원
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지 도 교 수　　이 재 련

이 논문을 의학박사 학위 논문으로 제출함

2022 년　2 월

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Abstract

Immune checkpoint inhibitors (ICIs) such as anti-programmed-death-1 (PD-1) and cytotoxic T lymphocyte protein-4 (CTLA4) inhibitors, are important treatment options for patients with advanced renal cell carcinoma (RCC). The tumor microenvironment (TME) in ICI-treated patients remains largely unknown. In this study, 71 patients were treated with ICIs between July 2015 and June 2020: 20 patients were treated with nivolumab plus ipilimumab as first-line therapy and 51 patients were treated with nivolumab monotherapy as second/third-line therapy. Multiplexed immunohistochemistry (mIHC) was performed to define the TME, which included various T cell subsets, B cells, macrophages, and dendritic cells. In the nivolumab plus ipilimumab group, the objective response rate (ORR) and disease control rate (DCR) were 47.1% and 64.7%, respectively. The density of CD8+ cytotoxic T cells (P=0.027), specifically CD103+ CD8+ tissue-resident T cells (P=0.046), CD137+ CD8+ T cells (P=0.005), Foxp3- CD4+ helper T cells (P=0.025), Foxp3+ CD4+ regulatory T cells (P=0.014), and CD68+ CD206- M1 macrophages (P=0.007) were significantly higher in responders than in non-responders. Patients who experienced initial

disease progression had a lower infiltration of CD68+ CD206- M1 macrophages than those who did not (P=0.008). The median progression-free survival (PFS) was 11 months, and a high density of CD8+ cytotoxic T cells (P=0.030), Foxp3- CD4+ helper T cells (P=0.036), CD137+ CD8+ cytotoxic T cells (P=0.024), and CD68+ CD206- M1 macrophages (P=0.054) was associated with better PFS. In the nivolumab group, ORR and DCR were 38.8% and 61.2%, respectively, and median PFS was 6.3 months. Response to nivolumab monotherapy was not significantly associated with the infiltration of T cell subsets and B cells, but patients who had initial disease progression with nivolumab had a higher infiltration of CD68+ CD206+ M2 macrophages than those who did not (P=0.012). A high density of CD137+ CD8+ cytotoxic T cells (P=0.016), CD137+ CD4+ cytotoxic T cells (P=0.088), and a low density of CD68+ CD206+ M2 macrophages (P=0.067) were associated with better PFS. When quantifying the infiltration of immune cells according to the International Metastatic RCC Database (IMDC) risk groups, CD68+ CD206+ M2 macrophages were more highly infiltrated in high-risk patients than in intermediate- or favorable-risk patients. The density of T cell subsets significantly correlated with that of CD68+ CD206- M1 macrophages and

CD20+ B cells, and the density of PD-L1+ cells significantly correlated with that of various immune cells. Therefore, through comprehensive TME analysis, CD137-expressing T cell subsets and M1/M2 macrophages were significantly associated with ICIs efficacy. This suggests that CD137 could be a predictive marker for selecting treatment options, and novel strategies targeting CD137 or macrophages should be further explored in advanced RCC.

Keywords: Renal cell carcinoma, tumor microenvironment, immune checkpoint inhibitors, response

Contents

List of tables and figures

INTRODUCTION

In recent decades, the prognosis of advanced renal cell carcinoma (RCC) has considerably improved due to the introduction of immune checkpoint inhibitors (ICIs), which block programmed death (ligand) 1 (PD-L1) or cytotoxic T lymphocyte antigen-4 (CTLA4). Following the phase III CheckMate-214 trial, nivolumab (an anti-PD-1 inhibitor) plus ipilimumab (an anti-CTLA4 inhibitor), applied as first-line therapy, improved objective response rate (ORR) (42% vs. 27%, $P<0.001$) and overall survival (OS) (hazard ratio [HR] 0.63, $P<0.001$) compared to sunitinib in intermediate- and poor-risk patients [1]. The phase III CheckMate-025 trial demonstrated better efficacy and safety with nivolumab in patients with advanced RCC after the failure of one or two regimens of anti-angiogenic therapy compared with everolimus (ORR 25% vs. 5%, $P<0.001$ and HR for OS 0.73, $P=0.002$) [2, 3]. However, only a limited number of patients benefit from ICIs. Approximately 20% (83/425) of the intermediate- and poor-risk patients from the CheckMate-214 trial [1] and 35% (143/410) of the patients from the CheckMate-025 trial [2] , respectively, experienced initial disease progression and had relatively short progression-free survival (PFS).

Currently, there are no biomarkers for predicting treatment response. The predictive and prognostic significance of PD-L1 expression, genomic mutations, tumor mutation burden, and gene expression patterns have previously been explored in ICIs-

treated patients [4-7]. However, understanding the determinants of treatment response is challenging. Given that the tumor microenvironment (TME) could influence the response to ICIs, investigation of the heterogeneous characteristics of TME is necessary to predict response, and a better understanding of underlying patients' immunity could suggest novel strategies to further improve clinical outcomes [8, 9].

In the present study, we performed multiplexed immunohistochemistry (mIHC) to investigate the features of TME in patients with advanced RCC receiving ICIs and evaluated the prognostic implications for the prediction of treatment response.

MATERIALS AND METHODS

1. Patients

Figure 1 shows the consort diagram of the patients included in this study. A total of 98 patients with advanced RCC were treated with ICIs as first- or second/third-line therapy at Asan Medical Center, Seoul, Republic of Korea, between July 2015 and June 2020. Among them, evaluable 71 patients had available tissues for mIHC, nivolumab plus ipilimumab as first-line therapy (n=20), and nivolumab monotherapy as second/third-line therapy (n=51). After quality testing of the tissue specimens, mIHC was performed in 66 patients in the final. This study was approved by the Institutional Review Board of Asan Medical Center (study number: 2019-1712), and it was conducted in accordance with the Declaration of Helsinki and Good Clinical Practice.

Patients with International Metastatic RCC Database (IMDC) [10] intermediate- or poor-risk could receive nivolumab (3 mg/kg) and ipilimumab (1 mg/kg) intravenously as first-line therapy every 3 weeks in four doses, followed by nivolumab (3 mg/kg) every 2 weeks. Nivolumab was administered (3 mg/kg) intravenously every 2 weeks as

second/third-line therapy in case of no prior use of ICIs. Since 2019, nivolumab could be also administered 6 mg/kg intravenously every 4 weeks or as a fixed dose of 240 mg every 2 weeks or 480 mg every 4 weeks [11, 12]. Tumor response was assessed using computed tomography every 6 to 9 weeks for the first year and then every 9 to 12 weeks until disease progression or discontinuation of ICI treatment, based on the response evaluation criteria in solid tumors (RECIST) criteria v1.1 [13].

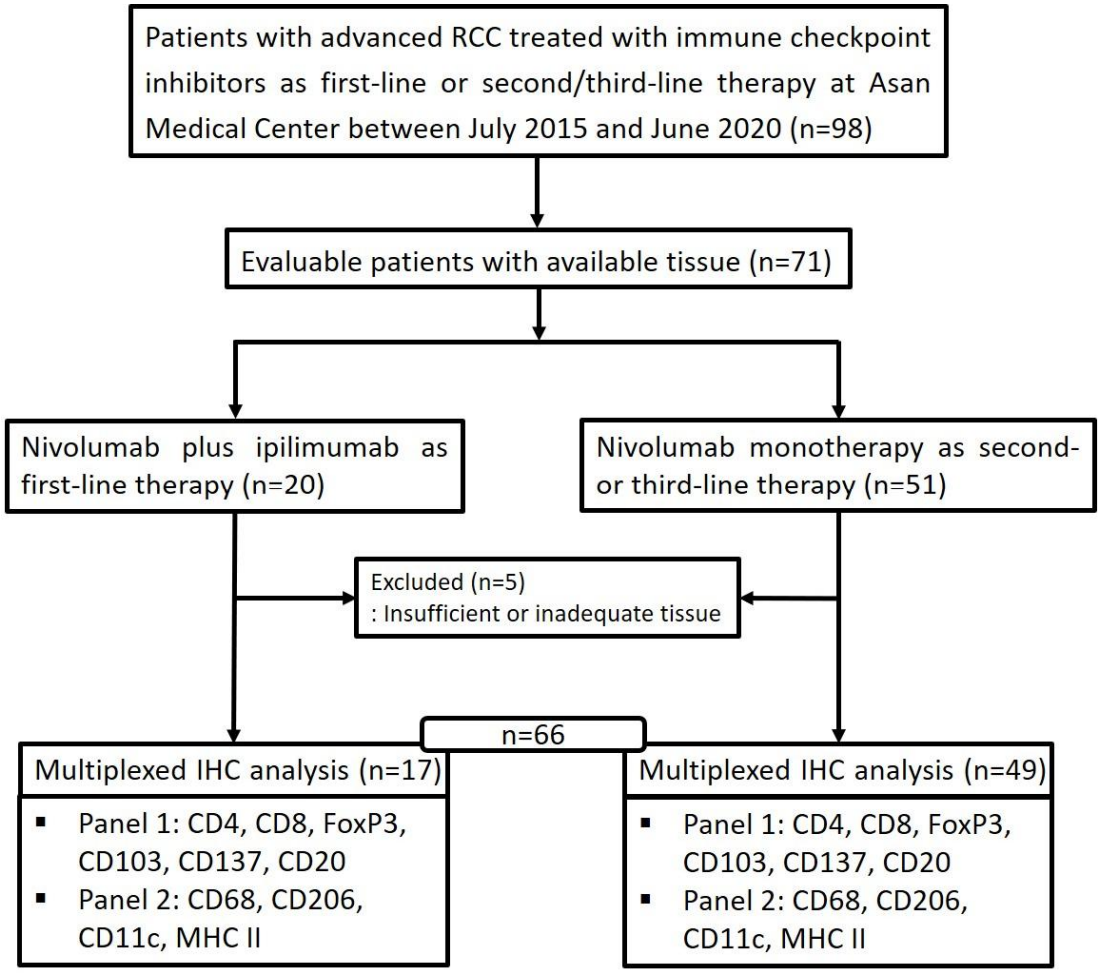


Figure 1. Consort diagram.

Abbreviation: RCC; renal cell carcinoma, IHC; immunohistochemistry

2. Multiplexed immunohistochemistry

Optimized fluorescent mIHC was performed by tyramide signal amplification (TSA) using a Leica Bond Rx™ Automated Stainer (Leica Biosystems, Newcastle, UK). Cells were stained with antibodies against CD20 (ab9475; Abcam, Cambridge, UK), CD4 (ab133616; Abcam), CD103 (ab129202; Abcam), Foxp3 (ab20034; Abcam), CD137 (ab197942; Abcam), CD8 (MCA1817; Bio-Rad, Hercules, CA, USA), CD206 (NBP1-90020; Novus Biologicals, Littleton, CO, USA), CD68 (ab 192847; Abcam), CD11c (ab52632; Abcam), MHCII (ab 7856; Abcam), and PD-L1 (13684S; Cell Signaling Technology, Danvers, MA, USA), and the fluorescence signals were captured with the following fluorophores: Opal 480, Opal 520, Opal 570, Opal 620, Opal 690, and Opal 780. Multiplex-stained slides were obtained using the Vectra® Polaris Quantitative Pathology Imaging System (PerkinElmer, Boston, MA, USA). The images were analyzed using inForm 2.4.4 image analysis software (PerkinElmer) and Spotfire™ software (TIBCO Software Inc., Palo Alto, CA, USA).

Regions of interests (ROIs) representing each tissue specimen were carefully

chosen by pathologists specializing in RCC based on hematoxylin and eosin slides, and we selected approximately 7–11 ROIs for each tissue specimen. Representative images are shown in Figure 2, and the implications for each marker are explained in Table 1. CD8+ was used to indicate cytotoxic T cells; CD103+ CD8+ for tissue-resident T cells and CD137+ CD8+ or CD137+ CD4+ for costimulatory 4-1BB-expressing T cells, both used as activated T cells; Foxp3- CD4- for helper T cells; Foxp3+ CD4+ for regulatory T cells; CD20+ for B cells; CD206- CD68+ for M1-polarized macrophage; CD206+ CD68+ for M2-polarized macrophage; CD11c+ for dendritic cells; MHCII+ for antigen presentation cells; and PD-L1+ for immune regulatory molecules. Cell numbers are expressed as the mean/mm^2 for each cell population.

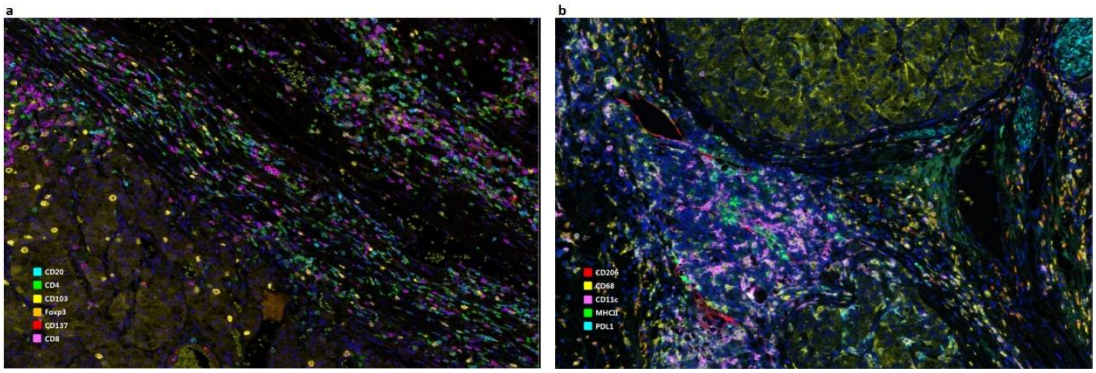


Figure 2. Representative examples of multiplexed immunohistochemistry staining of advanced renal cell carcinoma. a CD20, CD4, CD103, Foxp3, CD137, CD8; b CD206, CD68, CD11c, MHCII, PDL1. x 200 magnification for all.

Table 1. Implications for each marker of multiplexed immunohistochemistry.

Antibody used in panel 1	Implication
CD8	Found on the surface of cytotoxic T cells
CD4	Found on the surface of helper T cells or regulatory T cells
Foxp3	Functions as a master regulator of the regulatory pathway in the development and function of regulatory T cells
CD103	Found on the surface of intraepithelial lymphocyte T cells
CD137	Costimulatory activity for activated T cells and found on the surface of CD8+ and CD4+ lineages
CD20	Found on all stages of B cell development and functions for increased B cell immune response
Antibody used in panel 2	
CD68	Found on the surface of all macrophages, including M1 and M2 phenotypes
CD206	Found on the surface of macrophage and distinguishes M1 or M2 phenotypes
CD11c	Found on the surface of dendritic cells
MHCII	Found on the surface of professional antigen-presenting cells
PD-L1	Found on the surface of tumor cells or immune cells

3. Statistical analyses

Categorical and quantitative data were compared using the chi-square test or Fisher's exact test, and Mann–Whitney U tests. Mean levels of the markers among the three groups were compared using analysis of variance (ANOVA); multiple comparison tests were not performed. PFS was calculated from the date of ICIs initiation to the date of disease progression or death from any cause, whichever occurred first. The OS was calculated from the date of ICIs initiation to the date of death from any cause. Survival was estimated using the Kaplan–Meier method, and the log-rank test was used to compare the differences between the curves. A two-sided *P*-value <0.05 was considered significant, and all statistical analyses were performed using the statistical package for the social sciences (SPSS) 25.0 software package (IBM SPSS Statistics, Chicago, IL, USA).

RESULTS

1. Patient characteristics

A total of 66 patients who underwent mIHC analysis were included in this study (Figure 1). The baseline patient characteristics are summarized in Table 2. The median patient age was 62 years (range, 33–88 years), and 77.3% of the patients were men. Among the 66 patients, 17 (25.8%) received nivolumab plus ipilimumab as first-line therapy and 49 (74.2%) received nivolumab monotherapy as second/third-line therapy. The available tissues were obtained before ICIs treatment. Although fresh tissues just before ICIs treatment were used for mIHC analysis in an attempt to appropriately reflect TME just before ICI treatment, there were differences in the time from tissue acquisition to the start of ICI treatment (median; 3.7 months vs. 16.0 months between the nivolumab plus ipilimumab and nivolumab groups). Tissues were obtained from surgery (n=14) or biopsy (n=3) in the nivolumab plus ipilimumab group, and from surgery (n=30) or biopsy (n=19) in the nivolumab group.

Table 2. Baseline characteristics.

	Total patients (n=66, %)	**Nivolumab plus ipilimumab (n=17, %)**	**Nivolumab (n=49, %)**
Median age, years (range)	62 (33−88)	62 (39−76)	63 (33−88)
Sex			
male	51 (77.3)	13 (76.5)	38 (77.6)
female	15 (22.7)	4 (23.5)	11 (22.4)
IMDC risk group			
Favorable	7 (10.6)	0 (0)	7 (14.3)
Intermediate	38 (57.6)	11 (64.7)	27 (55.1)
Poor	21 (31.8)	6 (35.3)	15 (30.6)
Histology type			
Clear cell*	57 (86.4)	16 (94.1)	41 (83.7)
Presence of sarcomatoid component	17 (25.8)	6 (35.3)	11 (22.4)

Site of metastasis			
Lymph node	33 (50.0)	8 (47.1)	25 (51.0)
Lung	55 (83.3)	14 (82.4)	41 (83.7)
Liver	9 (13.6)	3 (17.6)	6 (12.2)
Bone	29 (43.9)	7 (41.2)	22 (44.9)
Treatment line			
First- line	17 (25.8)	17 (100)	–
Second-line	23 (34.8)	–	23 (46.9)
Third-line	26 (39.4)	–	26 (53.1)
Previous nephrectomy	51 (77.3)	14 (82.4)	37 (75.5)

Abbreviation: ECOG PS; Eastern Cooperative Oncology Group Performance Status

*Nine patients had non-clear cell type: unclassified (n=3), MiT translocation (n=3), papillary type 2 (n=2), and tubulosolid (n=1).

Table 3 summarizes the efficacy of ICIs. In the nivolumab plus ipilimumab group, the ORR and disease control rate (DCR) were 47.1% and 64.7%, respectively. At a median follow-up duration of 11.9 (95% confidence interval [CI], 8.4–15.3) months, the median PFS was 11.0 (95% CI, 0.0–22.6) months; median OS was not reached because only five (29.4%) patients died at the time of analysis. In the nivolumab group, ORR and DCR were 38.8% and 61.2%, respectively. At a median follow-up duration of 29.5 (95% CI, 26.8–32.1) months, the median PFS and OS were 6.3 (95% CI, 0.0–12.9) months and 18.9 (95% CI, 6.9–31.0) months, respectively.

Table 3. Clinical response and survival with ICIs.

	Nivolumab plus ipilimumab (n=17, %)	Nivolumab (n=49, %)
Response		
Complete response	2 (11.8)	1 (2.0)
Partial response	6 (35.3)	18 (36.7)
Stable disease	3 (17.6)	11 (22.4)
Progressive disease	6 (35.3)	19 (38.8)
ORR	8 (47.1%)	19 (38.8%)
DCR	11 (64.7%)	30 (61.2%)
Median PFS	11.0 (95% CI, 0.0-22.6) months	6.3 (95% CI, 0.0-12.9) months
Median OS	Not reached	18.9 (95% CI, 6.9-31.0) months

Abbreviations: ORR, objective response rate; DCR, disease control rate; PFS, progression-free survival; OS, overall survival

2. Association of the density of T cell subsets and B cells with responses to ICIs

Due to the different types of ICIs, the density of T cell subsets was compared between responders (complete response [CR] + partial response [PR]) and non-responders within each ICI treatment group. The number of immune cells in the TME of advanced RCC is listed according to the response in Table 4. In the nivolumab plus ipilimumab group, the density of CD8+ cytotoxic T cells (*P*=0.027), Foxp3- CD4+ helper T cells (*P*=0.025), and Foxp3+ CD4+ regulatory T cells (*P*=0.014) was significantly higher in responders than in non-responders (Figure 3a-3c). Specifically, CD103+ CD8+ tissue-resident T cells (*P*=0.046) and CD137-expressing T cells (CD137+ CD8+ T cells; *P*=0.005 and CD137+ CD4+ T cells; *P*=0.092) were highly infiltrated in responders compared with non-responders (Figure 3d-3f). The density of CD20+ B cells was numerically higher in responders than in non-responders (*P*=0.135). Patients who experienced initial disease progression had a lower infiltration of Foxp3- CD4+ helper T cells than those who did not (*P*=0.078) (Figure 3g). In contrast, response to nivolumab monotherapy was not significantly associated with the infiltration of T cell subsets and B cells.

Table 4. Infiltration of immune cells in responders and non-responders.

	Nivolumab plus ipilimumab (n=17, %)		Nivolumab (n=49, %)	
	Responders (n=8), median (IQR 25%-75%)	Non-responders (n=9), median (IQR 25%-75%)	Responders (n=19), median (IQR 25%-75%)	Non-responders (n=30), median (IQR 25%-75%)
CD8+ cytotoxic T cell	458.13 (203.63-862.70)*	107.81 (50.12-328.28)*	306.25 (102.73-438.44)	179.61 (74.61-544.73)
CD103+ CD8+ tissue-resident T cell	24.69 (16.45-280.12)*	13.44 (4.38-27.27)*	39.84 (16.80-93.75)	48.52 (8.41-138.95)
CD137+ CD8+ T cell	2.03 (1.50-12.77)*	0.0 (0.0-1.13)*	27.34 (3.13-42.19)	6.17 (0.86-37.50)
Foxp3- CD4+ helper T cell	483.29 (298.60-929.48)*	61.50 (23.52-233.52)*	495.05 (294.69-664.06)	466.25 (252.77-940.55)
Foxp3+ CD4+ regulatory T cell	19.38 (7.46-30.16)*	0.78 (0.39-10.55)*	5.21 (1.88-15.16)	7.58 (0.60-16.29)
CD137+ CD4+ T cell	4.14 (1.37-29.66)	0.31 (0.00-5.99)	44.92 (0.94-142.97)	32.81 (2.54-80.66)
CD20+ B cell	33.44 (13.05-106.02)	5.31 (1.17-54.30)	12.11 (1.56-60.31)	10.31 (1.60-29.18)
CD68+ CD206- M1 macrophage	643.67 (408.95-1148.24)*	126.50 (71.59-575.16)*	503.28 (258.10-712.89)	612.34 (391.72-978.98)

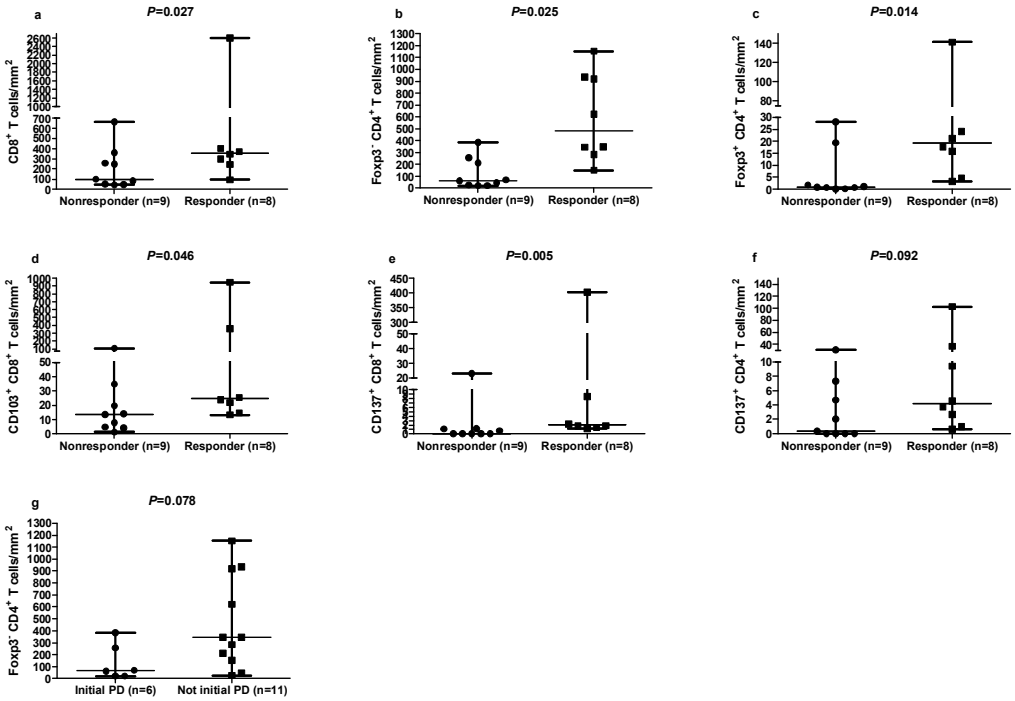
CD68+ CD206+ M2 macrophage	3.67 (1.10-12.46)	0.63 (0.0-2.42)	2.03 (0.59-10.16)	5.39 (0.63-42.42)
PD-L1+ CD68+ CD206- M1 macrophage	303.05 (184.57-449.41)	73.20 (29.06-374.95)	168.44 (148.05-255.31)	245.70 (106.02-578.16)
PD-L1+ CD68+ CD206+ M2 macrophage	5.63 (0.66-14.34)	0.31 (0.0-2.26)	1.95 (0.47-10.00)	2.27 (0.43-26.18)
CD11c+ MHC class II+ dendritic cell	0.39 (0.0-5.43)	0.0 (0.0-0.08)	0.0 (0.0-0.16)	0.0 (0.0-0.04)
PD-L1+ CD11c+ MHC class II+ dendritic cell	0.31 (0.0-5.00)	0.0 (0.0-0.0)	0.0 (0.0-0.16)	0.0 (0.0-0.04)
PD-L1+ cells	612.97 (439.80-1017.27)	382.29 (74.69-875.16)	361.88 (268.67-641.95)	429.69 (82.71-1042.46)

Abbreviation: IQR; interquartile

Cell numbers are expressed as the mean /mm2 for each cell population.

* $P<0.05$ by Mann-Whitney U-test

Figure 3. Association between T cell subsets and response with nivolumab plus ipilimumab. a. CD8+ cytotoxic T cells, b. Foxp3- CD4+ helper T cells, c. Foxp3+ CD4+ regulatory T cells, d. CD103+ CD8+ tissue-resident T cells, e. CD137+ CD8+ T cells, and f. CD137+ CD4+ T cells.

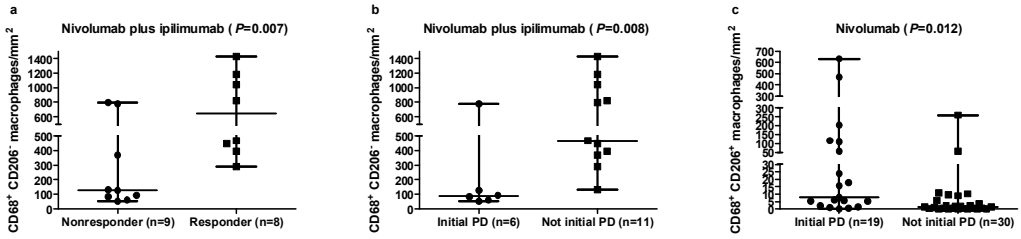


Abbreviation: PD; progressive disease

3. Association of the density of macrophages and dendritic cells with responses to ICIs

High infiltration of CD68+ CD206- M1 macrophages was significantly associated with achieving response to nivolumab plus ipilimumab (P=0.007) (Figure 4a), while its low infiltration was significantly associated with initial disease progression (P=0.008) (Figure 4b). In the nivolumab group, patients who experienced initial disease progression had a higher infiltration of CD68+ CD206+ M2 macrophages than those who did not (P=0.012) (Figure 4c). Otherwise, there were no significant differences in the density of dendritic cells and MHCII or PD-L1-expressing immune cells between responders and non-responders, regardless of the ICI type.

Figure 4. Association between M1/M2 macrophages and response or initial disease progression with ICIs. a. CD68+ CD206- M1 macrophage with nivolumab plus ipilimumab, b. CD68+ CD206+ M2 macrophage with nivolumab.

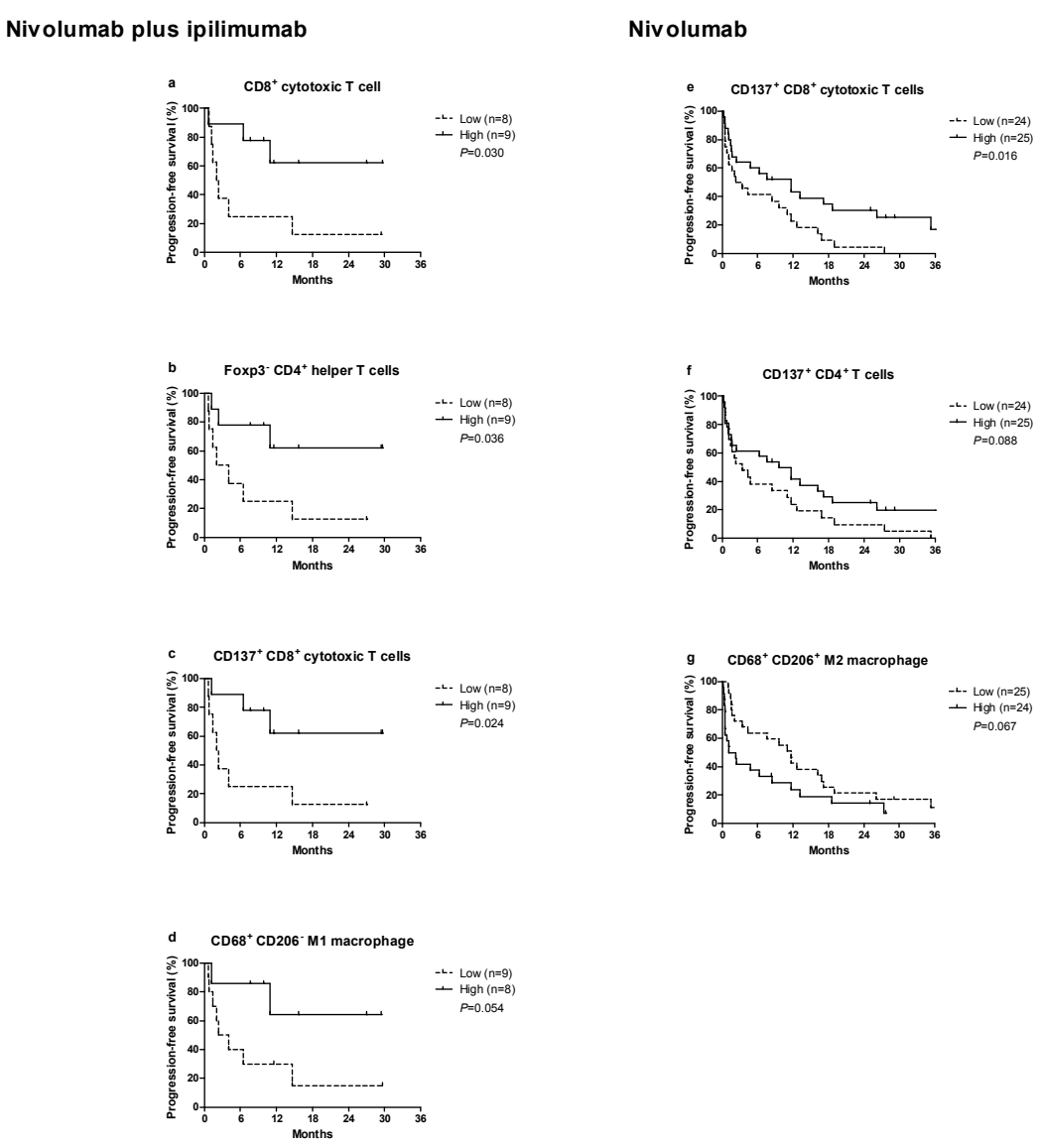


Abbreviation: PD; progressive disease

4. Association of TME with PFS and ICIs

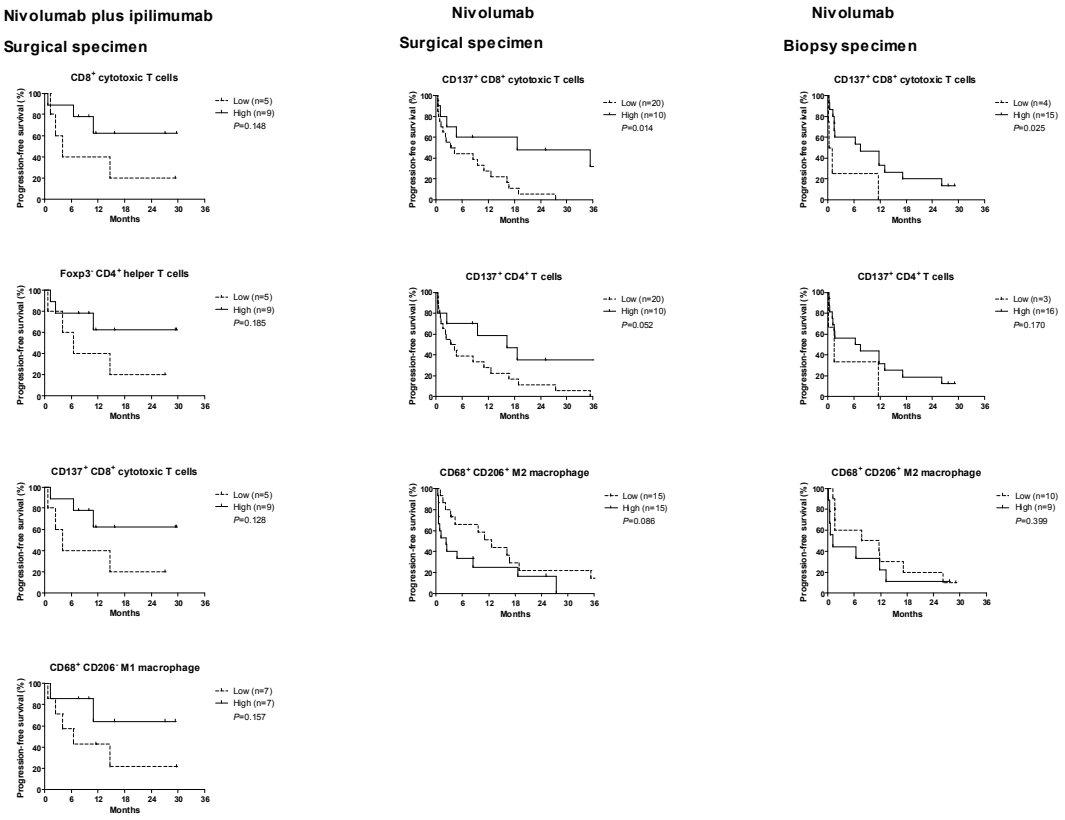
Each TME markers were classified into high (≥median) and low (<median) groups. The high density of CD8+ cytotoxic T cells (*P*=0.030), Foxp3- CD4+ helper T cells (*P*=0.036), and CD137+ CD8+ cytotoxic T cells (*P*=0.024) was significantly associated with better PFS with nivolumab plus ipilimumab (Figure 5a-5c). Despite the non-significant association of the density of CD20+ B cells with the response to nivolumab plus ipilimumab, their high density was significantly associated with better PFS (*P*=0.023). A high density of CD68+ CD206- M1 macrophages was marginally associated with better PFS with nivolumab plus ipilimumab (*P*=0.054) (Figure 5d). In the nivolumab group, patients with a higher density of CD137+ CD8+ cytotoxic T cells also had a significantly better PFS (*P*=0.016) (Figure 5e). The high density of CD137+ CD4+ cytotoxic T cells (*P*=0.088) and low density of CD68+ CD206+ M2 macrophages (*P*=0.067) were marginally associated with better PFS with nivolumab (Figure 5f and 5 g).

Figure 5. Progression-free survival with ICIs according to the density of T cells and macrophages.



We also examined the association between PFS and the density of immune cells in different types of tissue specimens (surgical vs. biopsy specimens) (Figure 6). Despite the limitation in evaluating statistical significance with a small number of subgroup analyses, especially within the nivolumab plus ipilimumab group, the association between the above TME biomarkers and treatment response appeared important regardless of tissue type. Given that there were only three patients with biopsy specimens in the nivolumab plus ipilimumab group, PFS with nivolumab plus ipilimumab according to TME in these patients could not be analyzed.

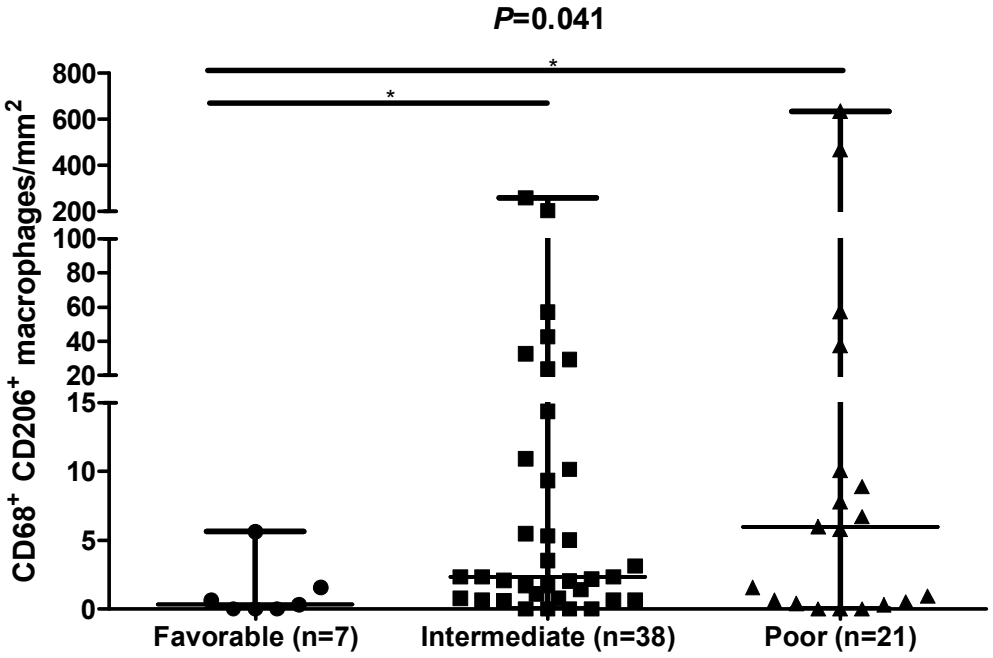
Figure 6. Progression-free survival with ICIs according to the density of T cells and macrophages in each surgical or biopsy specimen.



5. TME characteristics

Immune cell infiltration was quantified according to the IMDC risk groups. The density of CD68+ CD206+ M2 macrophages was significantly higher in IMDC intermediate- or high-risk patients than in favorable-risk patients (P=0.041 by ANOVA) (intermediate-risk vs. favorable-risk; P=0.012 and poor-risk vs. favorable-risk; P=0.028) (Figure 7). With numerical differences between intermediate- and high-risk patients (P=0.150), the density of CD68+ CD206+ M2 macrophages was the highest in IMDC high-risk patients (Figure 7). The density of T cell subsets showed mild or moderate but significant correlations with those of CD68+ CD206- M1 macrophages and CD20+ B cells (Figure 8). The density of PD-L1+ cells was significantly correlated with that of the various immune cells (Figure 9).

Figure 7. M2 macrophage infiltrations according to IMDC risk groups.



* $P<0.05$

Abbreviation: IMDC; International Metastatic Renal cell carcinoma Database

Figure 8. Correlation between density of T cells and M1 macrophages or B cells.

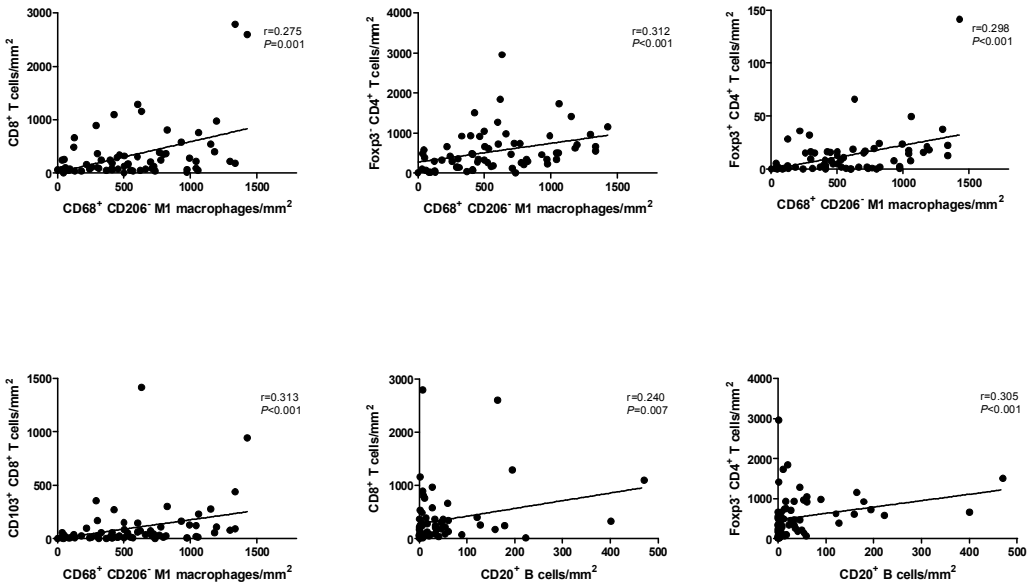
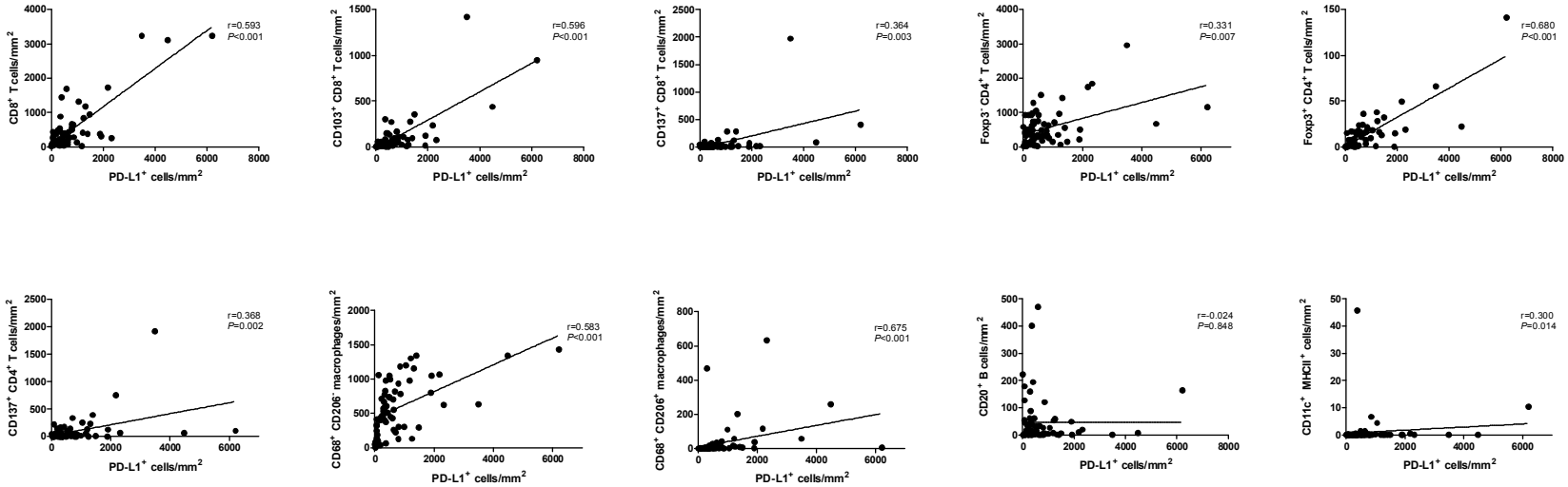


Figure 9. Correlation between immune cells and PD-L1+ cells.



DISCUSSION

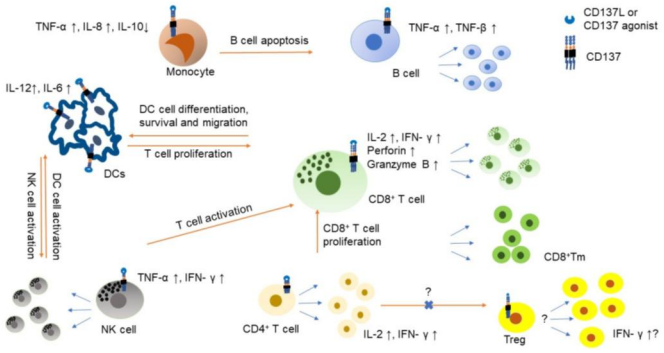
The current study showed a significant association between TME and response or PFS to ICIs through mIHC analysis. Importantly, a high density of CD137-expressing T cells and M1 macrophages, or a low density of M2 macrophages, were associated with better PFS. The response to nivolumab plus ipilimumab was further associated with various T cell subsets, B cells, and macrophages, while the response to nivolumab monotherapy was not. Patients who experienced initial disease progression had a lower density of M1 macrophages and a higher density of M2 macrophages than those who did not. CD68+ CD206+ M2 macrophages were more highly infiltrated in the IMDC high-risk patients than in the intermediate- or favorable-risk patients, and the density of PD-L1+ cells showed significant correlations with those of various immune cells. To the best of our knowledge, this is the unique study to comprehensively investigate the TME, focusing on specific CD137-expressing T cell subsets and macrophages and their clinical implications in ICI treatment settings.

1. Previous biomarker studies using data from the pivotal trials

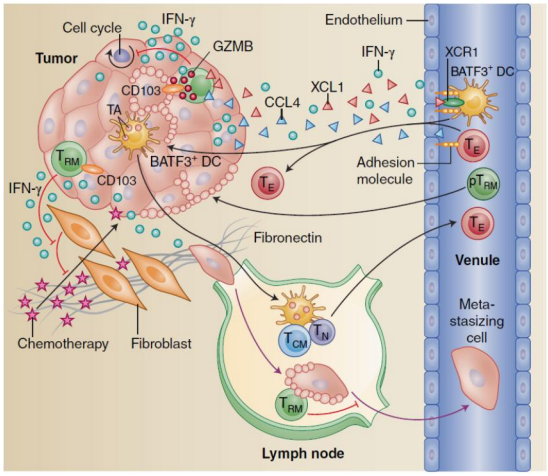
Exploratory biomarker studies [4-7] using pivotal trials, including CheckMate-214 and CheckMate-025, have been conducted to predict ICI treatment responses. In the CheckMate-214 trial, PD-L1 IHC, whole exome sequencing, and RNA sequencing were performed to evaluate PD-L1 positivity, tumor mutation burden, indel burden, human leucine antigen class I zygosity, PBRM1 mutation status, and gene signature scores [4]. Although tumor mutation burden and genomic instability could serve as robust predictors of ICI response in various cancers, these expected factors, as well as PD-L1 positivity, were not associated with the clinical benefit of nivolumab plus ipilimumab [4]. Only the inflammatory response and epithelial-mesenchymal transition (EMT) gene signatures have been linked with prolonged PFS [4]. In the CheckMate-025, -010, and -009 trials, tumor mutation burden and CD8+ T cell infiltration were not predictive of nivolumab monotherapy [5-7]. Recently, gene expression profiles [14, 15] using RNA sequencing of data from the IMmotion 150 and IMmotion 151 trials, comparing atezolizumab (anti-PD-L1 inhibitor) alone or in combination with bevacizumab (anti-vascular endothelial growth factor inhibitor) compared to sunitinib, revealed seven subtypes of advanced RCC. Among them, patients with $Teff^{High}$ had improved PFS with atezolizumab plus bevacizumab compared to sunitinib (HR 0.76, 95% CI 0.59-0.99), and $Teff^{High}$ subgroups were associated

with increased expression of PD-L1 on immune cells by IHC and with increased CD8+ T cell infiltration [14, 15]. Unlike previous studies, the present study directly examined various immune cells in tissues, which are major players in the TME associated with antitumor activity. Moreover, our mIHC approach enhanced the quality of TME analysis, considering that the difference between certain T cell subsets is not detectable by conventional IHC.

2. Clinical implications of cytotoxic T cells and specifically activated T cells



[CD137 expression on immune cells]

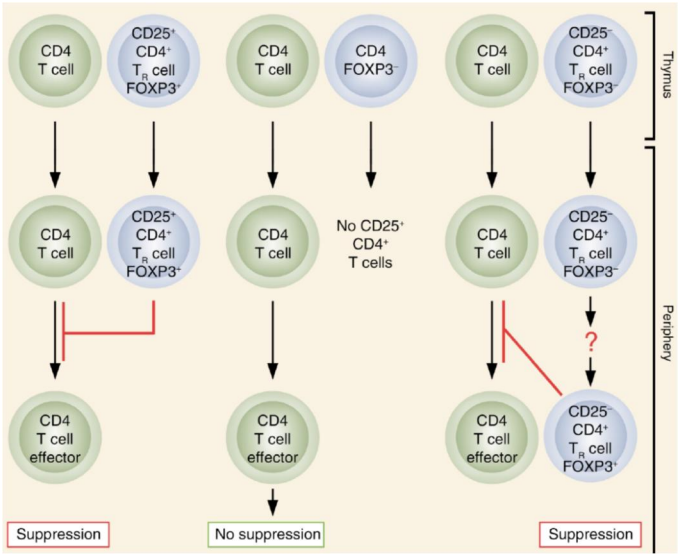


[CD103$^+$ tissue-resident T cells]

There is growing interest in unraveling the role of TME in identifying biomarkers, and exploring the heterogeneity of TME is complicated in highly immune-infiltrated RCC [8, 9]. Simple measurement of CD8+ T cells is unlikely to be predictive of ICI response [8], and defective T cell function in RCC has been reported in several studies [16-18]. This indicates that activated T cell subsets could be determinants of ICI responses in advanced RCC. We found that CD137+ CD8+ T cells, as a population of activated T lymphocytes, were significantly more infiltrated in responders than in non-responders, suggesting that CD137 could represent a predictive biomarker of response to ICIs. Similarly, we also found that the density of CD103+ CD8+ tissue-resident T cells, recently considered as activated and tumor-specific T cells within the tissue [19], was significantly higher in responders than in non-responders in the nivolumab plus ipilimumab group, although no significant

differences in PFS were observed. It is well known that signaling through CD137 induces the activation of CD8+ T cells, enhancing T cell survival, promoting their effector function, and favoring memory differentiation [20]. Moreover, we found that a high density of CD137+ CD4+ T cells was marginally associated with response to nivolumab plus ipilimumab (P=0.092) and PFS with nivolumab monotherapy (P=0.088). Numerous studies have shown that although both activated CD4+ and CD8+ T cells express CD137, signals through CD137 are more biased toward CD8+ T cells [21-23] and the main tumor-killing dependent on CD137 stimulation arises from CD8+ T cells [24]. In addition to the prognostic value of CD137, the efficacy and safety of CD137 agonists alone or in combination with ICIs have been investigated in several studies [25-27]. A phase Ib study of utomilumab (CD137 agonist) in combination with pembrolizumab (an anti-PD1 inhibitor) in patients with advanced cancers reported that six patients (26.1%) achieved confirmed responses, including CR (n=1), PR (n=1), stable disease (n=2), and progressive disease (n=1) in five patients with RCC [25]. Novel therapeutic strategies targeting the upregulation of CD137 expression or enhancement of CD137 signaling for synergistic effects with ICIs need to be further studied in advanced RCC.

3. Clinical implications of helper T cells and regulatory T cells

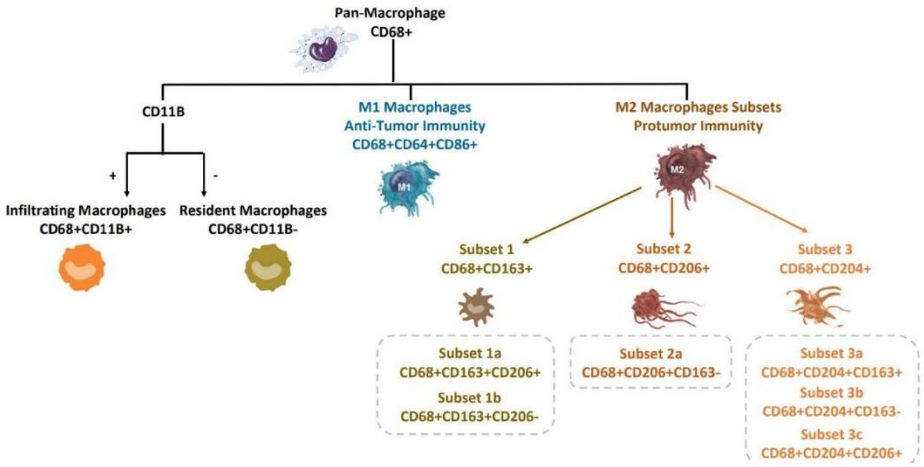


[Foxp3 expression on CD4$^+$ T cells]

Emerging evidence suggests that CD4+ T cells may also play a critical role in immune responses. Foxp3- CD4+ helper T cells are known to promote the priming of tumor-specific CD8+ T cells and help elicit durable T cell responses by interacting with dendritic cells in an MHCII-dependent manner [28]. In line with this, the density of Foxp3- CD4+ helper T cells was significantly associated with response and PFS with nivolumab plus ipilimumab. Regarding Foxp3+ CD4+ regulatory T cells known to play opposite roles in antitumor immunity [28], we found that the density of Foxp3+ CD4+ regulatory T cells was inversely higher in responders to nivolumab plus ipilimumab than in non-responders. This may be explained by the fact that the antitumor activity of anti-CTLA4 inhibitors is

dependent on the depletion of CTLA4-expressing regulatory T cells in the TME through antibody-dependent cellular cytotoxicity [29]; patients with a higher density of Foxp3+ CD4+ regulatory T cells can be more susceptible to anti-CTLA4 inhibitors. In fact, higher Foxp3+ CD4+ regulatory T cells at baseline have been reported to be significantly associated with favorable outcomes with ipilimumab in patients with melanoma [30].

4. Clinical implications of tumor-associated macrophages



[CD68 and CD206 on macrophages]

Tumor-associated macrophages consist of the M1 and M2 phenotypes. Based on CD68 and CD206 IHC, CD68+ CD206- M1 macrophages participate in antigen presentation and inflammation, while CD68+ CD206+ M2 macrophages are related to immunosuppression and tumor progression such as tumor growth, metastasis, and treatment

resistance [31]. In a meta-analysis of metastatic RCC, patients with a high density of M2 macrophages had poor PFS (HR 1.40, 95% CI 1.14-1.72) and OS (HR 1.32, 95% CI 1.16-1.50) [31]. In the present study, responders to nivolumab plus ipilimumab had higher infiltration of M1 macrophages than non-responders, and patients who experienced initial disease progression had a lower density of M1 macrophages or higher density of M2 macrophages than those who did not. There were also trends toward better PFS in patients with a high density of M1 macrophages or low density of M2 macrophages, respectively.

Interestingly, we found that the density of M2 macrophages was the highest in IMDC high-risk patients, and novel strategies for manipulating tumor-associated macrophages could be considered to overcome the poor prognosis of these patients. One of the mechanisms involved in the suppression of antitumor immunity mediated by macrophages is the depletion of essential metabolites such as tryptophan for T cell proliferation [32]. Based on the finding that indolamine 2,3 dioxygenase 1 (IDO1) overexpressed by M2 macrophages depletes tryptophan, which hampers T cell proliferation [32], the combination of epacadostat (IDO1 inhibitor) and pembrolizumab (anti-PD-1 inhibitor) showed promising responses, with an ORR of 47% in 19 patients with advanced

RCC previously treated with antiangiogenic agents, irrespective of risk groups [33]. Furthermore, the combination of epacadostat and ipilimumab also showed a promising ORR of 23% in immunotherapy-naïve melanoma patients [34]. The efficacy of the combination of epacadostat with ICIs needs to be further investigated, focusing only on intermediate- or high-risk RCC patients or a triple combination of epacadostat with nivolumab plus ipilimumab in advanced RCC.

5. Different results between nivolumab plus ipilimumab and nivolumab groups

TME seemed to be more associated with response or PFS with nivolumab plus ipilimumab than with nivolumab alone. Although the reasons for this are not clear, the time of tissue acquisition may be a major contributor to the results. The possibility of harboring different TME profiles cannot be excluded in the case of archival tissues obtained not just before second/third-line nivolumab because prior exposure to sunitinib or pazopanib (anti-angiogenesis) could modify the TME in these patients. There are also several potential reasons for this. In previous gene expression profiles, $Teff^{High}$ was more frequent among patients with intermediate- or poor-risk, and sarcomatoid RCC was enriched in the T

effector or proliferative types [14, 15]. The Teff^{High} subtype, considered to have a more pre-existing intratumoral adaptive immune response, could be enriched in the nivolumab plus ipilimumab group due to the initial intermediate- or poor-risk patients (100%) and the presence of a sarcomatoid component (35.3%), leading to more correlations between TME and efficacy.

6. Limitations and future directions

The present study had some limitations. First, only a small number of patients treated with nivolumab plus ipilimumab were included. Since the CheckMate 214 trial, nivolumab plus ipilimumab has become an important option as first-line treatment; however, this regimen was of limited use because it was not covered by the National Health Insurance Service (NHIS) of Korea yet. Further, larger studies to confirm the value of significant TME biomarkers are required to provide solid evidence. Second, only approximately 30% and 50% of the patients died in the nivolumab plus ipilimumab and nivolumab groups, respectively, at the time of the analysis. After further follow-up, OS data could be analyzed. Third, TME

analysis through mIHC may not represent the entire tissue specimen because it is limited to ROIs; in particular, there are concerns when using biopsy specimens rather than surgical specimens. It may be necessary to investigate a wider area of tumor tissues for clinical use in daily practice, as well as to validate TME biomarkers associated with treatment response. We also showed an association between TME and efficacy of ICIs, regardless of tissue type. Fourth, there could be limitations of the mIHC analysis itself, such as the interaction between multiple markers and interference between multiple wavelengths used in mIHC analysis. Nevertheless, this novel quantitative multispectral imaging method has been validated to reflect conventional IHC-based immune cell evaluation [35] and is increasingly used to assess the immune profiles of the TME [36]. Its clinical usefulness deserves further investigation in future studies on various immune subsets and clinical settings.

We are currently conducting additional mIHC analyses to increase the number of patients in the same setting. In addition, we are working to develop a predictive model using TME biomarkers identified as promising in this study and to evaluate the spatial distribution of immune cell infiltration (tumor center vs. tumor margin vs. stroma). Follow-up research

is currently being planned to investigate the infiltration of inflammatory cells or epithelial-mesenchymal transition features using an additional panel of mIHC analysis in the same study patients. We plan to propose a prospective study evaluating the efficacy and safety of CD137 or macrophage-targeting strategies plus ICIs in patients with intermediate/poor-risk advanced RCC.

In conclusion, various immune cells in the TME are comprehensively associated with the response to ICIs. In particular, CD137-expressing T cell subsets and M1/M2 macrophages were significantly associated with ICI efficacy, suggesting novel predictive biomarkers and targets to further improve survival.

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국문요약

진행성 신장암에서 면역관문억제제 치료제는 현재 중요한 치료 옵션이 되었다. 하지만, 면역관문억제제를 치료한 환자들에서 종양미세환경에 대해서는 잘 알려진 바가 없다. 본 연구에서는 2015 년 7 월부터 2020 년 6 월 사이에 총 71 명의 면역관문억제제 치료를 받은 환자들을 포함하였고, 일차 치료로서 nivolumab plus ipilimumab 치료를 받은 20 명과 이차 또는 삼차 치료로서 nivolumab 단독 치료를 받은 51 명이었다. 다중 면역조직화학검사는 다양한 T 세포, B 세포, 대식세포, 그리고 수지상 세포 등 종양미세환경을 파악하기 위해 시행하였다. Nivolumab plus ipilimumab 치료 그룹에서 종양 반응율과 조절율은 각각 47.1%, 64.7%였다. CD8+ T 세포 (*P*=0.027), 특히 CD103+ CD8+ T 세포 (*P*=0.046)와 CD137+CD8+ T 세포 (*P*=0.005), Foxp3-CD4+ T 세포 (*P*=0.025), Foxp3+CD4+ T 세포 (*P*=0.014), 그리고 CD68+CD206- M1 대식세포 (*P*=0.007)가 종양 반응을 획득한 환자에서 그렇지 못한 환자들보다 유의하게 높게 관찰되었다. 초기에 급격히 질병이 악화되는 환자들에서 CD68+CD206- M1 대식세포가 그렇지 못한 환자들보다 유의하게 적게 침윤되어 있었다 (*P*=0.008). 중위 무진행생존은 11 개월이었고, CD8+ T 세포 (*P*=0.030), CD137+CD8+ T 세포 (*P*=0.024), Foxp3-CD4+ T 세포 (*P*=0.036), 그리고 CD68+CD206- M1 대식세포 (*P*=0.054)의 높은 침윤이 더 향상된 무진행생존을 보였습니다. Nivolumab 치료 그룹에서 종양 반응율과 조절율은 각각 38.8%, 61.2%였고, 중위 무진행생존은 6.3 개월였다. Nivolumab 의 종양 반응은 T 세포, B 세포 침윤과 연관성이 없었으나, 초기에 급격히 질병이 악화되는 환자에서 CD68+CD206+ M2 대식세포가 그렇지 못한 환자들보다 유의하게 높게 침윤되어 있었다 (*P*=0.012). CD137+CD8+ T 세포 (*P*=0.024), CD137+CD4+ T 세포 (*P*=0.088)의 높은 침윤과 CD68+CD206+

M2 대식세포의 (*P*=0.067) 낮은 침윤은 더 향상된 무진행생존을 보였다. IMDC 위험도 그룹에 따라 면역세포 침윤을 살펴보면, CD68+CD206+ M2 대식세포가 저위험도 및 중간위험도 환자보다 고위험도 환자에서 더 많이 침윤하였다. 또한 다양한 T 세포 침윤과 CD68+CD206- M1 대식세포 및 B 세포 침윤사이에 연관성을 관찰하였고 PD-L1 양성 세포 침윤과 다양한 면역세포들의 침윤은 연관성이 있었다. 결론적으로, 포괄적인 종양미세환경 분석을 통해 CD137 을 발현하는 T 세포와 M1/M2 대식세포는 면역관문억제제의 효과와 유의한 연관성을 가진다. 이는 CD137 이 치료 선택에 있어서 예측인자가 될 수 있음을 시사하고 CD137 또는 대식세포를 활용한 새로운 치료 전략을 향후 진행성 신장암에서 연구할 필요성을 시사한다.